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THE CELL

Volume VI

Volume I	Part I: Methods Part II: Problems of Cell Biology
Volume II	Cells and Their Component Parts
Volume III	Meiosis and Mitosis
Volume IV	Specialized Cells, Part 1
Volume V	Specialized Cells, Part 2
Volume VI	Supplementary Volume

THE CELL

Biochemistry, Physiology, Morphology

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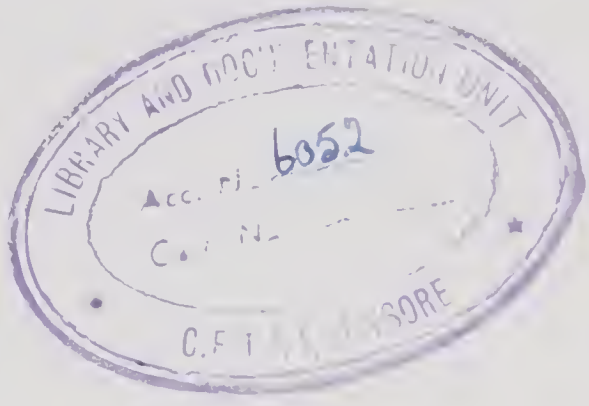

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PREFACE

"The Cell" has achieved such widespread and continued success that it encouraged the Editors and Publishers to add a sixth volume to the already existing series. This volume deals mainly with the cells of lower organisms; protozoa (nucleus and cytoplasm), fungi, and plant cells have been treated in greater detail than before, in the light of recent experimental discoveries concerning ultrastructure and biochemical activity. As a counterpart, the volume ends with chapters concerning highly specialized structures in higher organisms: collagen and sensory cells. In short, this volume is devoted to various aspects of cell differentiation which are as interesting and baffling in unicellular organisms as in collagen ultrastructure and synthesis, and in sensory receptors of mammals.

J. Brachet
A. E. Mirsky

January, 1964

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CHAPTER 1

The Protozoan Nucleus[1,2]

By KARL G. GRELL

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I. Introduction

Whereas older textbooks of cytology (Wilson, 1925; Belar, 1928; Geitler, 1934) paid considerable attention to the results of protozoological research, these subjects are barely mentioned in more recent compilations (White, 1954; De Robertis *et al.*, 1954; Swanson, 1957). One of the reasons for this is undoubtedly that it becomes increasingly difficult for any individual worker to encompass the whole field of cell research. A further reason may lie in the fact that protozoologists frequently have to cope with more difficult material, and their results may therefore be loaded with a higher degree of uncertainty. The present author, on the basis of many years of research on protozoan nuclei, is

[1] The systematics of Protozoa used corresponds to that in the author's "Protozoologie" (1956). This applies also to the terminology of stages in reproductive cycles.

[2] I wish to thank Dr. A. Ruthmann for translating the text and for valuable suggestions in its preparation.

however convinced that many aspects emerge from these studies which are important to the cytologist working with multicellular plants and animals. They may engender valuable comparisons with his own objects of study and may furthermore induce him to reevaluate his theoretical concepts. It is the aim of this article to foster this interest in protozoan cytology. Hence it is intended to present an introduction to the problematics of this field, rather than a compilation of our knowledge concerning protozoan nuclei.

No author, much as he may dislike to seem partial, can avoid giving more space to those objects and problems which are most familiar to him through his own work. We consider this natural and must appeal to the reader's indulgence.

Independent of the present review, diverse aspects of research on Protozoa are treated in the chapters by Mirsky and Osawa (Volume II), Brachet (Volume II), Mazia (Volume III), Nanney and Rudzinska (Volume IV) in this treatise.

II. The Structure of the Resting Nucleus

The nuclei of tissue cells of multicellular organisms are of rather uniform appearance. As a rule, chromosomes are not recognizable in them. Only chromocenters and nucleoli appear as stainable constituents, the former by virtue of their content of DNA, the latter through their RNA. Although there are nuclei of totally different structure—for example, nuclei with polytene chromosomes in the Diptera and endopolyploid nuclei of Hemiptera—these are exceptions as compared with the ubiquity of "chromocenter nuclei."

By contrast, chromocentric nuclei are absent in protozoa. Aside from two exceptions (Pätau, 1937; Grell, 1953b), heterochromatin, including sex chromosomes, is unknown in unicellular organisms. Yet the appearance of resting nuclei in Protozoa is determined to a high degree by the *variability of the chromosomal material.* There are two extremes in this respect, which are, however, connected by a graded series of intermediates.

One of the extremes is represented by "interphasic" resting nuclei, in which chromosomes are almost invisible. Nuclei of this type either do not stain with the Feulgen reagent at all or react only very weakly. Most phytomonads, protomonads, opalinids, amebae, heliozoans, acantharians, and sporozoans belong to this group.

Quite the opposite situation prevails in all those protozoan nuclei in which chromosomes are always recognizable as more or less pronounced threads. One would be tempted to call such intensely Feulgen-positive

nuclei "chromosomal" in contrast to "interphasic" resting nuclei, were it not certain that the chromosomes must retain their identity in both types regardless of appearance. Especially characteristic are nuclei of euglenoids and dinoflagellates in this respect (Fig. 2a), but similar appearances are found in many polymastigotes, radiolarians, and foraminiferans. The macronuclei of ciliates could also be included in this category (see Fig. 32).

It would seem likely that the differences in nuclear structure as outlined above are due largely to structural differences in the chromosomes themselves. All observations indicate that in Protozoa the chromosomes undergo changes of form and structure in connection with the coiling cycle similar to those of higher organisms. Very likely the chromosomes are quite uncoiled and extended in the "interphasic" type of resting nuclei and more or less tightly coiled in the "noninterphasic" ("chromosomal") type.

The limited resolving power of the light microscope does not permit one to verify this. Thus, it is not possible to distinguish the chromosomal material from the nuclear ground substance in "interphasic" resting nuclei. In highly condensed chromosomes of the noninterphasic type, the individual coils are not resolved microscopically.

Some recent electron microscope observations seem to be of great interest in this connection.

In "interphasic" resting nuclei of the amebae *Amoeba proteus* and *Pelomyxa carolinensis,* several researchers (Pappas, 1956a; Mercer, 1959; Pappas and Brandt, 1960; Roth *et al.*, 1960) found remarkable clusters of helices with 8–10 turns each and of about 8000 A. (0.8 μ) in length (Fig. 1). These helices are arranged around a central axis or core which could not be resolved into further structural detail. The helices themselves are 300–500 A. in diameter. More recently, Brandt and Pappas (1961) found indications of two 60–70 A. subunits within the filament composing the helix. Each of the subunits is a helix in itself, composed of a thread of about 20 A. diameter and thus corresponding to the dimensions of a Watson-Crick double helix. This may possibly represent one of the structural configurations in which interphase chromosomes can appear. It may correspond to the first step above the molecular level in the hierarchy of coiling systems. The interphase chromosome enters the next level of coiling when condensing during prophase.

However, this situation cannot be generally valid as similar clusters of helices so far have not been found in other interphase nuclei. On the other hand, it seems possible that the helices are completely unwound in such cases.

The question whether the condensation of the chromosomes in nuclei of the noninterphasic type can be ascribed to coiling is to be answered in the affirmative for the dinoflagellate *Amphidinium elegans* (Grell and Wohlfarth-Bottermann, 1957; Giesbrecht, 1961). Electron

FIG. 1. *Amoeba proteus*. Section from a portion of the Feulgen-positive region of the nucleus. Helical structures are arranged bristle-like around a central axis. The thickness of the filaments making up the coils is 120–150 A. After Pappas and Brandt (1960).

micrographs (Fig. 2b) show a distinct cross-banding of the chromosomes due to the helical course of fibrillar bundles. Individual fibrils are 30–80 A. thick. It is not known whether their length corresponds to that of the whole chromosome or whether there are shorter elements like the helices from ameban nuclei, which, however, would have to be completely unwound and should originate from some sort of longitudinal

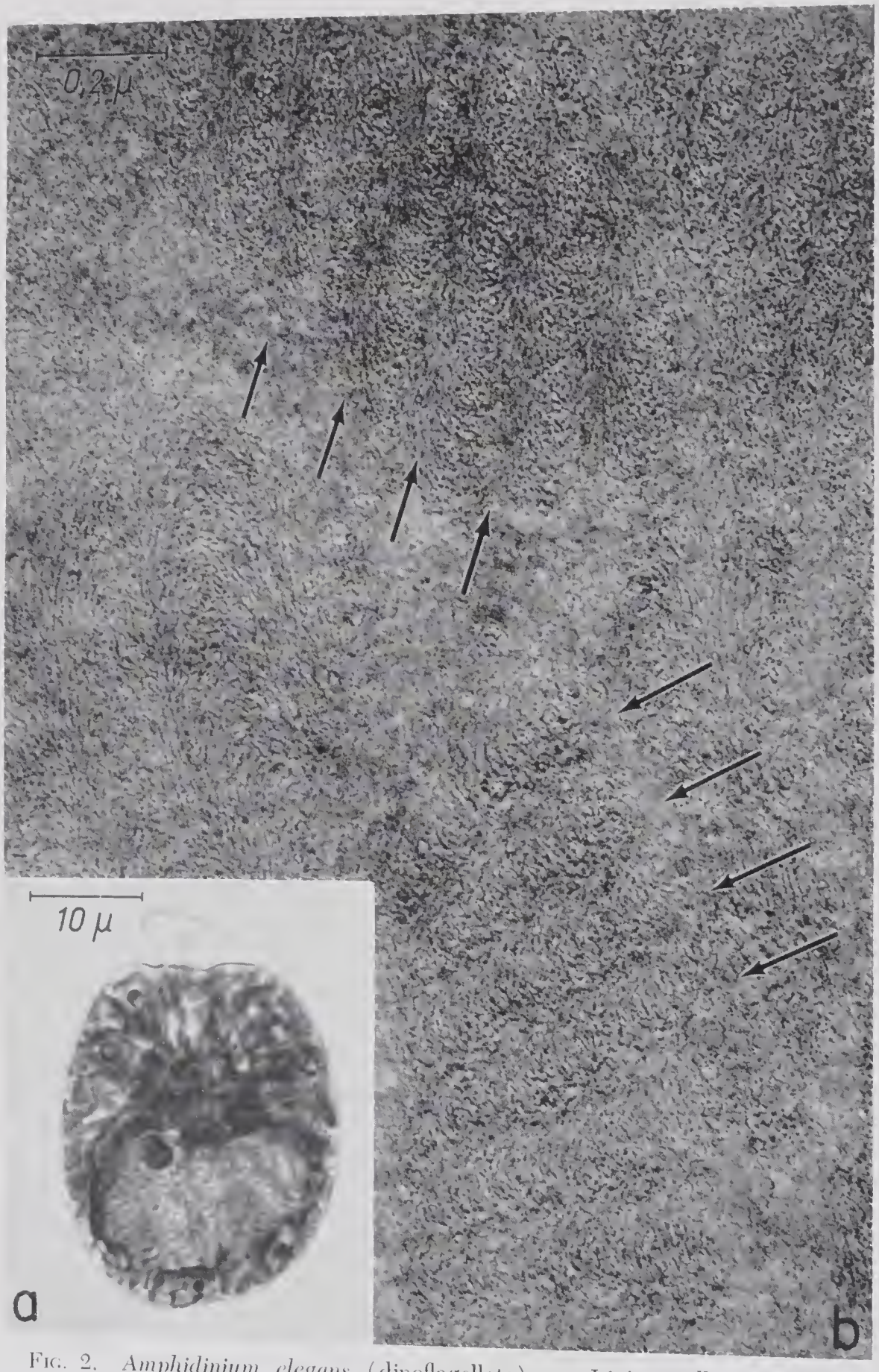


FIG. 2. *Amphidinium elegans* (dinoflagellate). a. Living cell; posterior part shows nucleus with chromosomes. b. Electron micrograph of a section through the nucleus; arrows point to multifibrillar coils of two chromosomes. After Giesbrecht (1961).

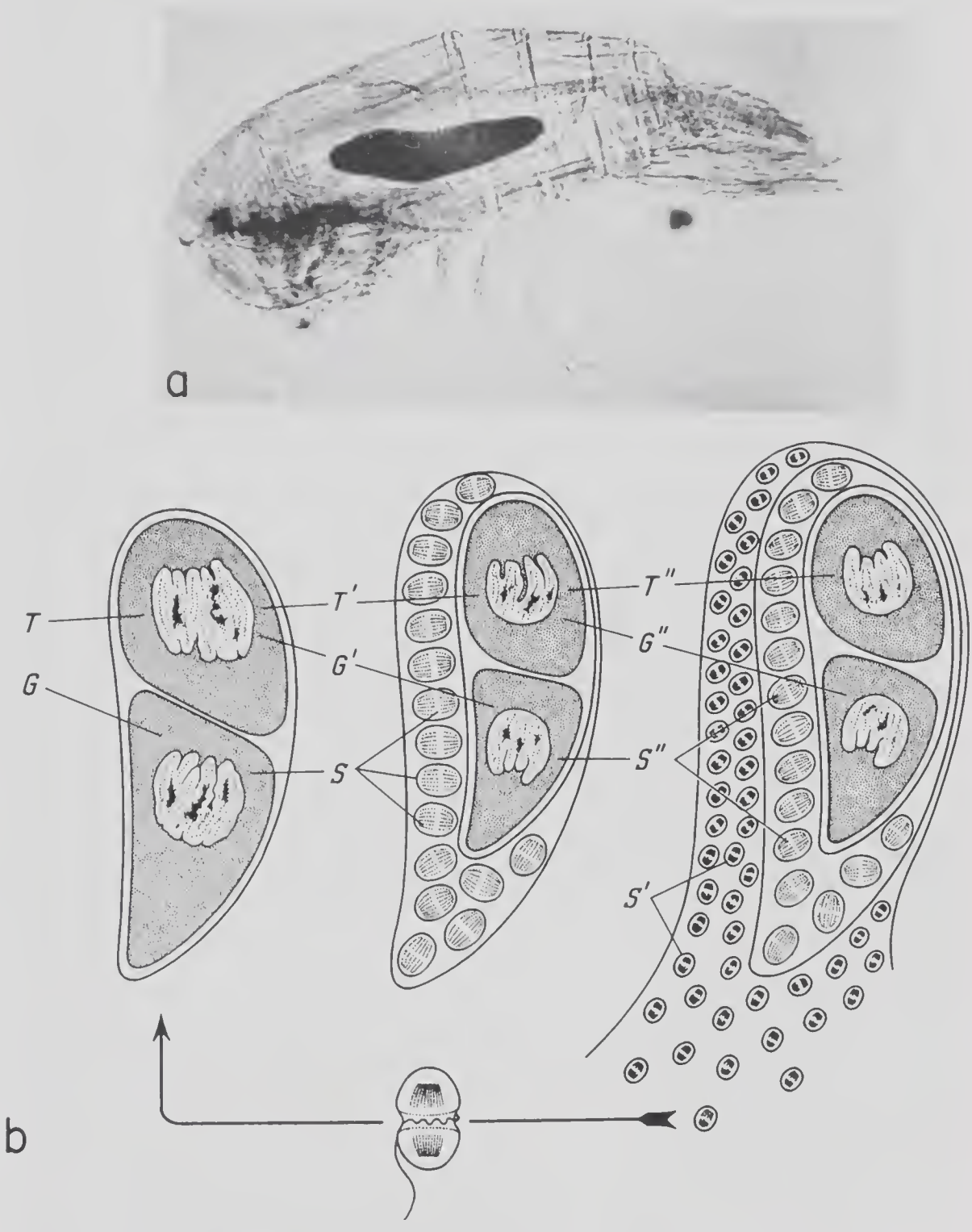


FIG. 3. *Blastodinium* (dinoflagellate). a. Germinative body in the midgut of a marine copepod (Feulgen stained). b. Developmental cycle of the germinative body (diagrammatic). Binucleated swarmer (bottom) grows and divides into two cells, the trophocyte (*T*) and the gonocyte (*G*). The trophocyte divides again, forming a new trophocyte (*T'*) and a new gonocyte (*G'*). In the meantime, the old gonocyte (*G*) has formed a first generation of presumptive swarmer cells. This process may be repeated several times, leading to concentric layers of presumptive swarmer cells representing successive generations. After Grell (1956).

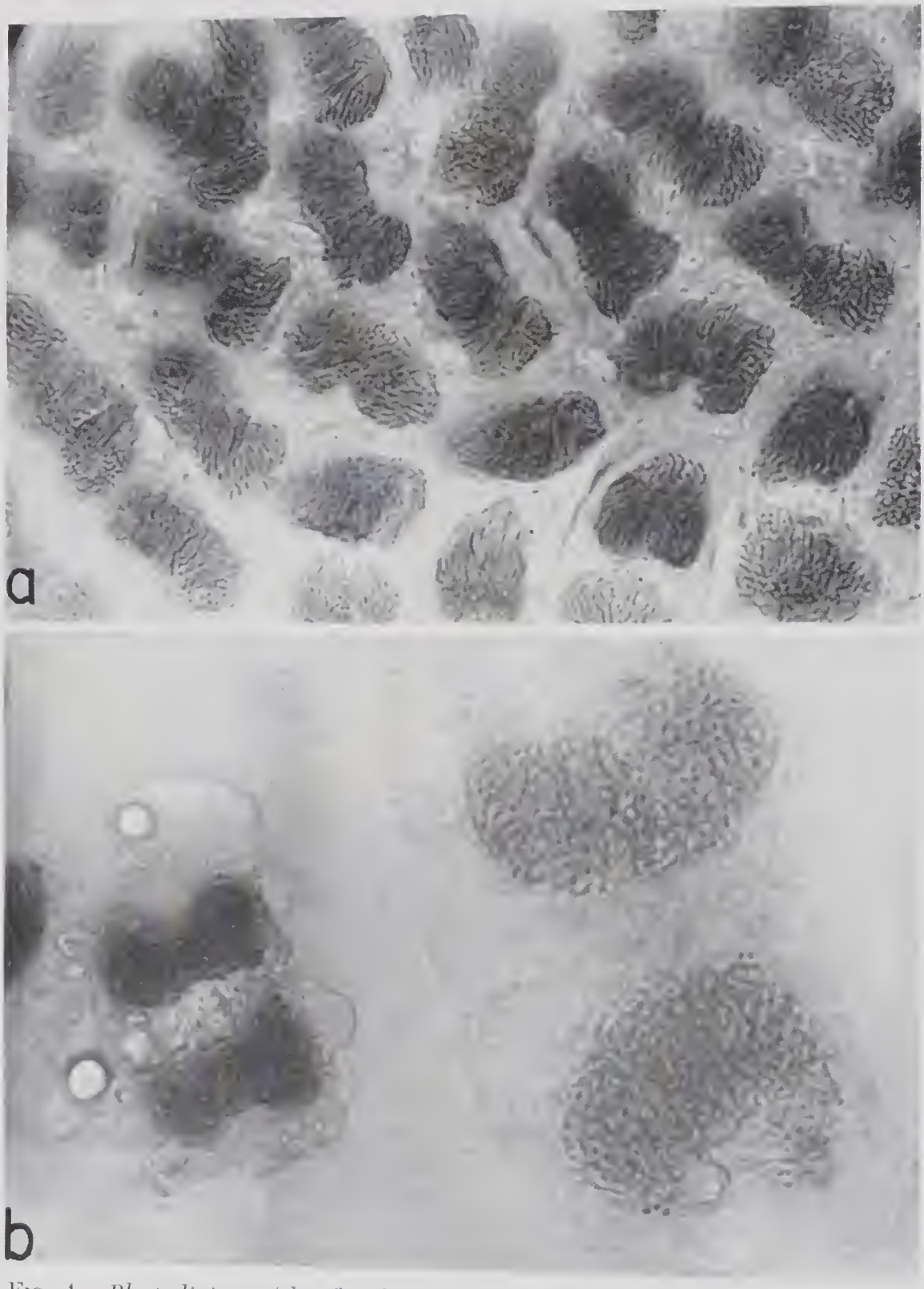


FIG. 4. *Blastodinium* (dinoflagellate). a. Presumptive swarmer cells of the same generation. b. Two presumptive swarmer cells of different generations. Both figures at same magnification. Stained with acetocarmine.

axis. Similar pictures of the fine structure of chromosomes have been described by Grassé and Dragesco (1957) from resting nuclei of other dinoflagellates.

Although of rather uniform appearance in general, the nuclei of dinoflagellates present us with one interesting example of continuous variation in the course of the developmental cycle of a species. Copepods of warm seas are often infected with dinoflagellates of the genus *Blastodinium* (Figs. 3 and 4). These enter the gut of copepods as binucleated swarmers. Intense growth and nuclear proliferation leads to large germinative bodies which release groups of swarmers at regular intervals (see Chatton, 1920, for details). It is of special interest in this context that nuclear structure in descendants of successive multiplication phases is characteristically different. At the beginning, the nuclei are large and almost Feulgen negative, with several elongated nucleoli. During successive proliferation periods, the nuclei become smaller. Simultaneously, they lose their nucleoli and become more and more Feulgen positive while their chromosomes continue to condense. As cell divisions occur more and more rapidly, the term "resting nuclei" is correspondingly harder to justify as development proceeds.

The different appearances of the resting nuclei of *Blastodinium* are not due primarily to changes in chromosomal morphology as characteristic of the divisional cycle. Hence, in this case, the chromosomes are subjected to a developmental life cycle which is superimposed upon phase-specific chromosomal changes connected with division. The latter are, as discussed above, entirely different in dinoflagellates in general and seem to play scarcely any role at all in *Blastodinium* specifically.

It is of interest in this connection that certain phases of nuclear division can be halted for extended periods of time while an actual "interphase" is missing. Such is the case with the hypermastigote flagellate *Holomastigotoides tusitala* (Cleveland, 1949a), whose nuclei are normally found in late prophase. In this stage, the chromosomes are already split into chromatids and are rather coiled (Fig. 5).

As a general result, we can state that physiological activity (which must be most intense in just those nuclei called "resting") is not bound to any specific state of chromosomal condensation. The nuclei of dinoflagellates with their rather condensed chromosomes are surely not less active than the "interphasic" resting nuclei of amebae.

Aside from the nuclear state as discussed above, the appearance of protozoan nuclei depends upon the number and size of the chromosomes. Species with high chromosome numbers like *Amoeba proteus* ($\sim$500) or the radiolarian *Aulacantha scolymantha* ($\sim$1500) have large

nuclei. In addition, the latter also has especially large chromosomes. The diameter of a nucleus of *Aulacantha scolymantha* can exceed 150 μ. In the ciliate *Paramecium bursaria*, the micronuclei of certain strains are of different sizes depending upon their degree of polyploidy (Chen, 1940a,b).

Such correlations between the size of the nuclei and the number of chromosomes are, of course, valid only if the chromosomal material is a major component of the total nuclear mass. Some types of giant nuclei owe their enormous size only to an increase in nuclear ground substance and nucleoli. An impressive example of this is furnished by

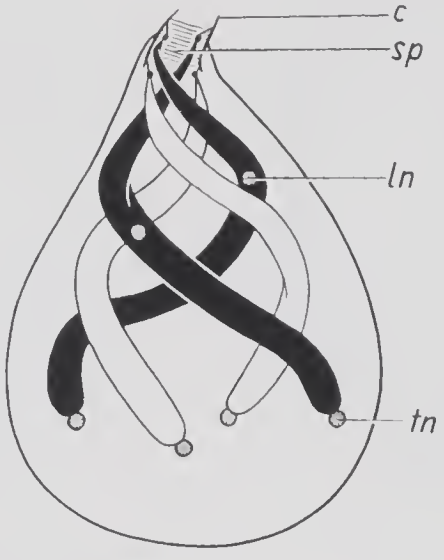


FIG. 5. *Holomastigotoides tusitala* (polymastigote flagellate). "Resting nucleus," cytologically a late prophase (see text). The chromatids of one chromosome are drawn in the same color. Black chromatids with a lateral (*ln*) and a terminal (*tn*) nucleolus. White chromatids with terminal nucleolus only. Both chromosomes are clearly telokinetic; rod-shaped centrioles (*c*) connected by spindle fibers (*sp*). After Cleveland (1953b).

the nuclei of many monothalamic foraminiferans. In *Myxotheca arenilega* we find rather few chromosomes in a central region of the nucleus, which consists largely of nuclear ground substance while the nucleolar material forms a dense layer just below the nuclear envelope. In the gamonts the beginning of gametogenesis is marked by a peculiar process of disintegration (Fig. 6). This affects first the nucleolar layer, resulting in the formation of large separate nucleoli which become irregularly distributed within the nuclear ground substance. The chromosomes move to the periphery of the nucleus, where a small spindle is formed. While the major portion of the giant nucleus is lost to the cytoplasm, two small daughter nuclei are formed around the poles of the spindle. These contain the same number of chromosomes as the former giant nucleus, but

much less nuclear ground substance and nucleolar material. The daughter nuclei multiply rather rapidly, and finally the whole cytoplasm is filled with nuclei destined to become the nuclei of the gametes. A similar nuclear disintegration is found in gregarines at the onset of gametogenesis.

Finally, attention should be drawn to the fact that the quantity and distribution of nucleolar material may also be responsible for the appear-

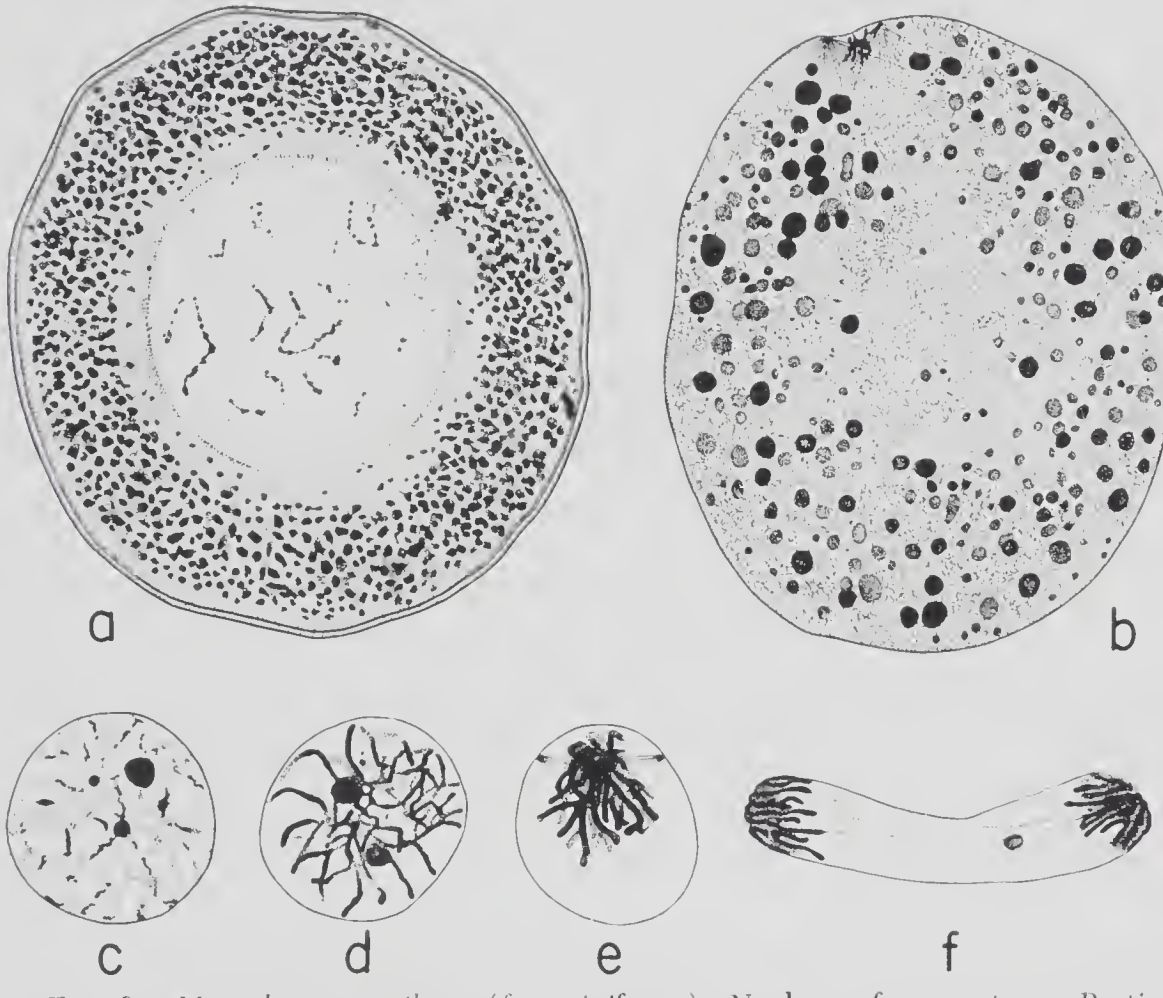


FIG. 6. *Myxotheca arenilega* (foraminiferan). Nucleus of gamont. a. Resting stage. b. First gamogony division. *c–f*. Later gamogony divisions. Magnification of a and b: × 1100; c–f: × 3000. After Föyn (1936).

ance of protozoan nuclei. One frequently encountered type is the "karyosome nucleus," which is characterized by a single centrally located nucleolus. It is found in "interphasic" (e.g., many amebae and sporozoa) as well as in "chromosomal" resting nuclei of euglenoids and foraminiferans. In other cases the nucleolar material is represented by many nucleoli at the nuclear periphery, forming occasionally distinct layers as in *Amoeba proteus*, the heliozoans *Actinophrys* and *Actinosphaerium*, and many monothalamic foraminiferans (Fig. 6a). More

rarely, nucleoli are present as elongated bodies, as described by Chen (1948) for opalinids of the genus *Zelleriella*. In this case, there is a clear relationship to speeific ehromosomes (nucleolus chromosomes). A similar situation prevails in hypermastigina of the genus *Holomastigotoides* (Cleveland, 1949a, 1953b) (see Fig. 5).

The nuclear membrane complex has been extensively investigated in *Amoeba proteus* (Bairati and Lehmann, 1952; Harris and James, 1952; Greider *et al.*, 1956, 1958; Pappas, 1956b; Mercer, 1959; Roth *et al.*, 1960). These electron microscope investigations have revealed the presenee of a honeyeomb structure immediately adjacent to the inner nuclear membrane. The "pores" of the nuclear envelope are in register with the honeycomb pattern below. However, the pores are of smaller diameter than the associated honeycombs. In *Endamoeba blattae* the order of struetural eomponents is reversed, the honeycombs appearing on the outside (Beams *et al.*, 1959). Investigators have considered that the thieker layer may serve to reinforce the nuclear envelope. In other protozoans, eonstruction of the nuelear border resembles that of higher plants and animals (e.g., Grimstone, 1959, in *Trichonympha*).

III. Some Features of Nuclear Division

It is not the aim of this chapter to present a detailed description of the different types of nuelear division in Protozoa (see Belar, 1926a; Grell, 1956). However, it seems useful to call attention to some facts and problems of general interest.

On the whole it can be stated that the variability of divisional modes is surprisingly large in Protozoa.

A. Mitosis

As far as mitosis is concerned, workers in this field have attempted to cover the wide spectrum of variability by inventing a whole gamut of terms (e.g., eumitosis, paramitosis, haplomitosis, dinomitosis, orthomitosis, pleuromitosis). In the author's opinion, this is neither desirable nor neeessary. While proper attention should be paid to the differences, it would seem more important to find a common denominator for all types.

1. Patterns of Mitosis

The wide variety of divisional types is largely due to differences in the *origin and formation of the mitotic apparatus.*

If the divisional centers are located within the cell nueleus, mitosis is called *intranuclear.* This is the case in many polymastigotes (e.g.,

Pyrsonympha, *Oxymonas*, *Saccinobaculus*, *Notila*), opalinids, and foraminiferans and also holds for the micronuclear division of ciliates. *Extranuclear* mitoses, on the other hand, are characterized by divisional centers outside the nuclear membrane. The latter must be at least partially dissolved to allow for connection of the spindle fibers with the kinetochores of the chromosomes.

The divisional centers themselves may be present as *polar caps*, representing calottes of denser cytoplasm which originate at the poles of the mitotic figure. Such polar cap mitoses are characteristic for higher plants. In Protozoa, they have been found only in two heliozoans, *Actinopsiaerium eichhorni* (Hertwig, 1898) and *Actinophrys sol* (Belar, 1922).

In most cases, the divisional centers of protozoa are characterized by the presence of distinct *centrioles* such as are found in multicellular animals. Occasionally, these are surrounded by a spherical plasma region, the *centrosome*, from which the polar rays originate.

In many cases, for instance in the gamogony divisions of gregarines (Fig. 7), the centrioles are free to move within the cytoplasm (movable centrioles). After duplication, the daughter centrioles migrate to opposite positions outside the cell nucleus. The nuclear envelope is subsequently more or less dissolved while the spindle is forming.

In polymastigotes, on the other hand, the centrioles occupy fixed positions within the cell (localized centrioles). Although these centrioles are commonly present as rodlike structures firmly anchored to the anterior end of the cell, they are found in various types with different life cycles (Cleveland, 1957b). As one example of this, we shall describe the situation in *Barbulanympha* (Fig. 8). In this genus the centrioles are especially large and can be clearly seen in the living animal. Ordinarily, each cell has two centrioles. Only its front end, which is somewhat swollen, is capable of autoreduplication at the onset of nuclear division, while the posterior portion is the only part engaged in the formation of spindle fibers. After division, the old centriole remains intact. Of the new centriole, the anterior swollen portion is formed first as a product of autoreduplication. The posterior portion arises by outgrowth, thus completing the normal set of two centrioles per cell.

Based on his observations of both normal and abnormal divisional stages, Cleveland could draw a number of conclusions that are of general cytological interest. Some of his results are summarized below without attention to details.

1. One centriole alone is incapable of forming a spindle.
2. Two centrioles can form a spindle even in the absence of a nu-

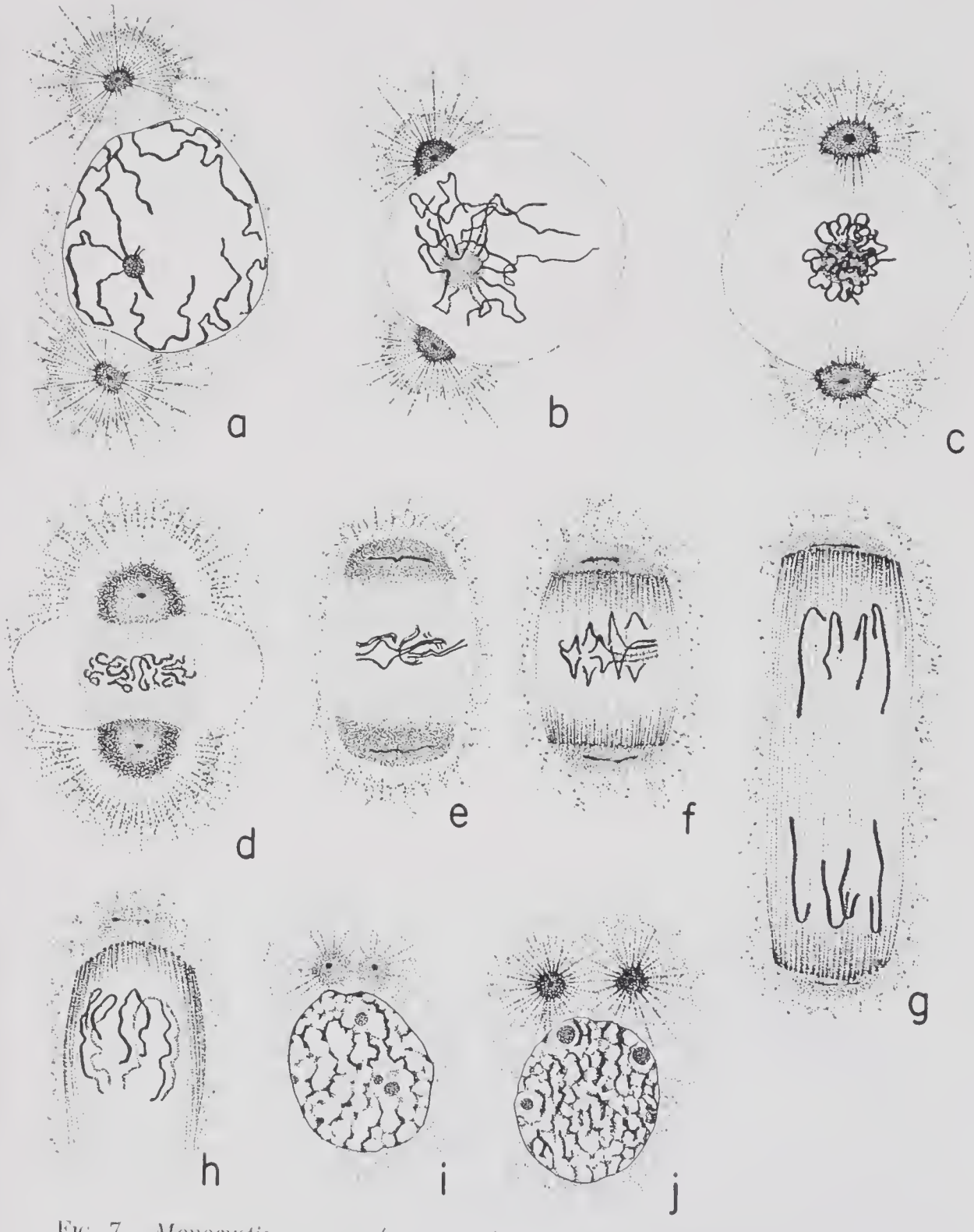


FIG. 7. *Monocystis magna* (gregarine). Progamic divisions. a, b. Prophase. c. Prometaphase. d. Metaphase. e–g. Anaphase. h–j. Telophase. Magnification: × 1850. After Belar (1926a).

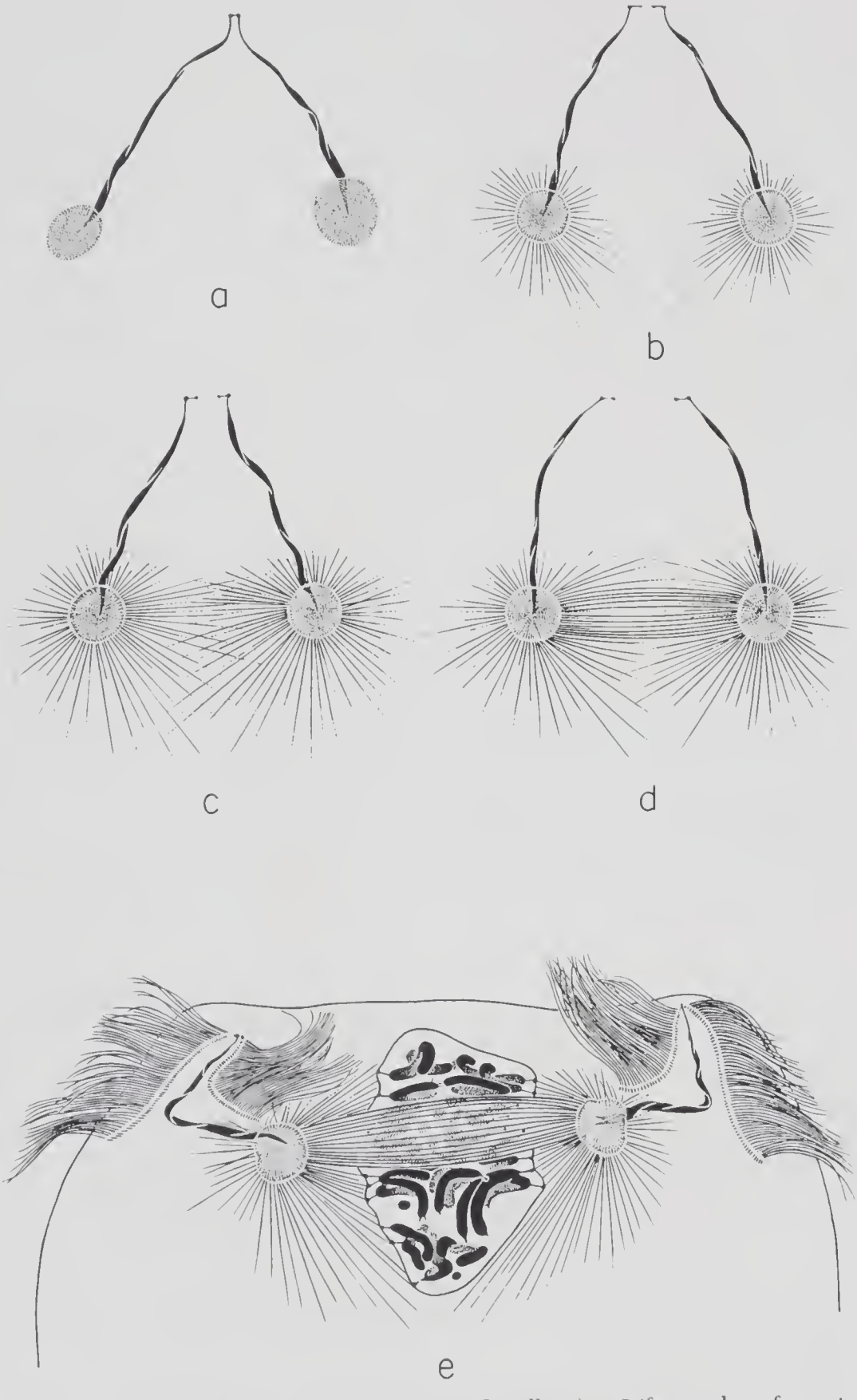


FIG. 8. *Barbulanympha* (polymastigote flagellate). Life cycle of centrioles (a–d). e. Anterior region of the cell during anaphase. After Cleveland (1938).

cleus or after destruction of the chromosomes within the nucleus by oxygen treatment.

3. Nuclear division is possible only if the polar rays which originate at the centrioles can reach the points of spindle fiber attachment of the chromosomes.

4. Depending upon suitable cytoplasmic conditions, the loss of centrioles is followed by endomitotic duplication of the chromosomes.

Besides autoreduplication and spindle formation, these centrioles function as *centers for organization* of many structures characteristic of polymastigotes, such as flagella, axostyles, parabasal bodies. Details are discussed in the papers of Cleveland (see mostly 1949–1956).

The appearance of protozoan mitoses may be varied not only through differences in the divisional apparatus, but also owing to the coiling cycles of chromosomes. Thus, during sporogony of the coccidian *Aggregata eberthi* divisions occur very rapidly, and it is not before telophase that the chromosomes achieve maximal condensation (Belar, 1926b).

Despite considerable variation in the pattern of nuclear divisions as discussed above, there is no doubt that we are dealing with genuine mitoses throughout. In all cases, the chromosomes reduplicate to form chromatids which are distributed by a special spindle apparatus to the daughter nuclei. It is also probable that the chromosomes are, in each case, provided with a special point of spindle fiber attachment (kinetochore, centromere).

2. *Problematical Cases*

The nuclear divisions to be discussed in this section present certain difficulties of interpretation. Thus, the presence of a spindle apparatus and of kinetochores is problematical, and it has even been suggested that we are not dealing with true mitoses in these cases.[3]

A. Euglenoidina, Dinoflagellata. Euglenoidina and Dinoflagellata may be treated together because the structure and division of their nu-

[3] Since equal distribution of two sets of chromosomes is the essence of mitosis, one might ask the question whether modes of nuclear division other than mitosis are theoretically possible. It is well known that the total gene complement of the bacterium *Escherichia coli* is contained within one identically reduplicating "chromosome." The distribution of both "chromatids" to the daughter cells takes place without any evident spindle apparatus. By analogy with *Escherichia coli*, it seems possible that some protozoans might contain a number of homologous chromosomes, each containing the whole set of genes. From a genetic standpoint, a spindle apparatus might be dispensed with in such cases, as there is no exacting requirement for the distribution of "chromatids" in nuclear division.

clei is similar in many respects. (1) Both have nuclei of the "noninterphasic" type (see page 2). In addition, most euglenoids have a central nucleolus. (2) Chromosomal numbers are always very high in both groups. (3) Chromosomes do not show the "normal" course of structural changes during nuclear division. (4) A spindle apparatus—in the sense of centrioles and a spindle between them—is lacking in both taxonomic groups. (5) In the course of division, the chromosomes of both euglenoids and dinoflagellates do not become arranged in an equatorial plate in the usual sense.

The analysis of chromosomal behavior in these groups is fraught with special difficulties, as the sequence of events cannot be deduced from the coiling cycle of the chromosomes. Thus, the chromosomes are frequently longest and thinnest at the mid-phase of nuclear division, and it is therefore scarcely possible to designate successive stages as prophase, metaphase, anaphase, and telophase.

The decisive point is, of course, whether or not a distribution of chromatids takes place as in ordinary mitosis. Early observations seemed to indicate that both chromatids move to the same pole. Of course, this would be possible only if all chromosomes were homologous. It would furthermore mean that the type of nuclear division found in euglenoids and dinoflagellates could not be regarded as a true mitosis. This possibility was considered by Grell (1953a) on the basis of earlier observations of other authors (Fig. 9).

Recent investigators have attempted to prove that these nuclear divisions can be interpreted in terms of the mitotic pattern. A thorough study on euglenoids was made by Leedale (1958a, b). He reached the conclusion that in this case mitosis is of the "nonspindle type" involving chromosomes without spindle fiber attachments. If the term were not already claimed for chromosomal fragments without kinetochores, one should have to call these chromosomes "akinetic," as they are neither monokinetic nor polykinetic. The absence of a spindle apparatus is further indicated by the finding that colchicine exerts no influence on chromosomal movements in the course of division. The doubling of chromosomes and the separation of chromatids are said to occur asynchronously and to be due to autonomous forces of the latter which lead to their mutual repulsion. Leedale thinks that the chromosomes are not homologous as he believes he has evidence for differences in chromosomal length in some cases and, in one of the species examined, for the existence of nucleolus chromosomes. Thus, he does not consider the interpretation proposed by Grell (1953a) as valid. However, it is difficult to reconcile Leedale's views with his finding of high percentages of

"amitosis" in some species of *Euglena* giving rise to nuclei with about half the number of chromosomes which were still able to carry out "mitosis" subsequently (Leedale, 1959).

In contrast to Leedale's observations, Saito (1961) maintains to see submedian, subterminal, or terminal kinetochores in the chromosomes of *Euglena viridis*. But his photomicrographs—though excellent—are not

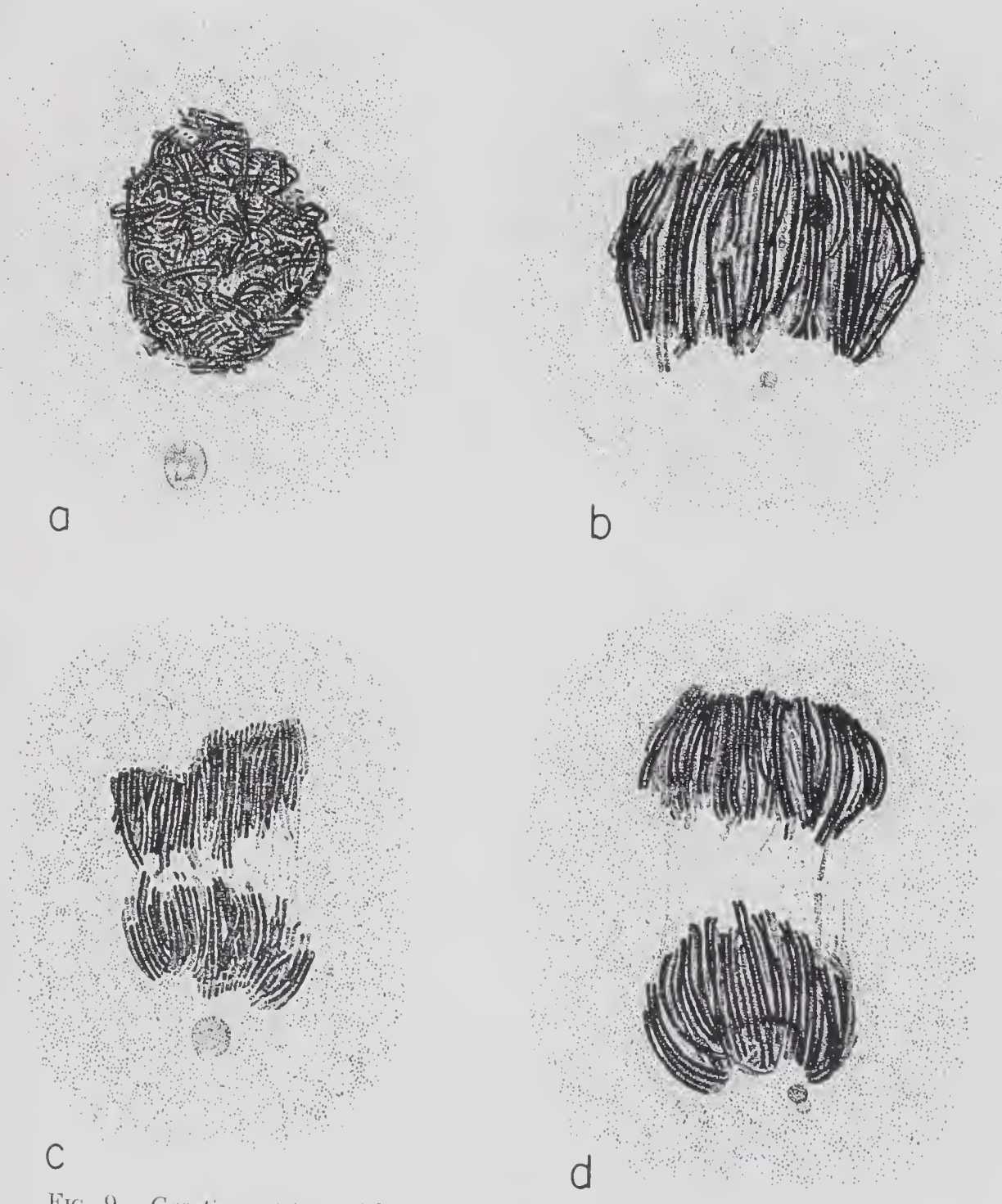


FIG. 9. *Ceratium tripos* (dinoflagellate). Stages of nuclear division. After Borgert (1910).

as convincing as his diagrams (Fig. 1A and B, in Saito, 1961). What they show with clarity is the relational coiling of chromatids.

The nuclear division of the dinoflagellate *Ceratium cornutum*, has recently been studied by Skoczylas (1958), who assumes the existence of a spindle without furnishing adequate proof. His conclusion is based on what he considers V-shaped chromosomes at prophase, indicating the existence of a kinetochore at the apex of the "V." At "metaphase," the kinetochores of all chromosomes are arranged along a ridge. According to this author, the whole difficulty in interpreting nuclear division figures is due to asynchrony in the separation of the chromatids.

One of the stages described by earlier authors (e.g., Borgert, 1910; see Fig. 9a) as "*Knäuelstadium*" (cluster stage) belongs, according to Skoczylas, to the meiotic sequence. However, there is no cytological evidence that we are dealing with a stage of meiosis in this case, especially, as the existence of sexual reproduction and hence of meiosis in dinoflagellates is extremely problematical. This stage is of special interest as it shows parallel chromatids, giving the impression of endomitosis.

Much as it would seem desirable to reconcile the events which characterize the division of euglenoid and dinoflagellate nuclei with the usual pattern of mitosis, the present reviewer holds that even the more recent work is not sufficient to firmly establish such deductions.

b. Radiolaria. In contrast to euglenoids and dinoflagellates, the radiolarians do not represent a taxonomically uniform group. Almost nothing is known about nuclear structure and division in whole suborders such as the Monopylea (Nassellaria) and Acantharia, the latter being separated by some authors (e.g., Trégouboff, 1953) from the remainder of the Radiolaria. Even in other radiolaria, only parts of the developmental cycle are known and have been investigated cytologically. The reason for this is that radiolarians occur only in marine plankton and that it has so far been impossible to culture them under laboratory conditions.

In order to provide a basis for evaluating the cytology of radiolarians, it is necessary to summarize briefly our knowledge on reproduction in this group.

In numerous species, two modes of formation of swarmers have been observed, called anisosporogenesis and isosporogenesis, respectively, by the earlier workers. Chatton (1920, 1923, 1934) and Hollande and Enjumet (1953, 1955) have succeeded in establishing that "anisosporogenesis" represents the formation of swarmers of parasitic dinoflagellates multiplying either outside or within the central capsule, or even at first exclusively within the radiolarian nucleus. The swarmers formed are

similar to *Gymnodinium* and are often of different sizes and shapes (hence "anisospores"). *Isosporogenesis*, which occurs mostly in the very same species leads to swarmers bearing crystalline inclusions (*"Kritall-schwärmer"*). It is generally agreed that the latter are true stages of the

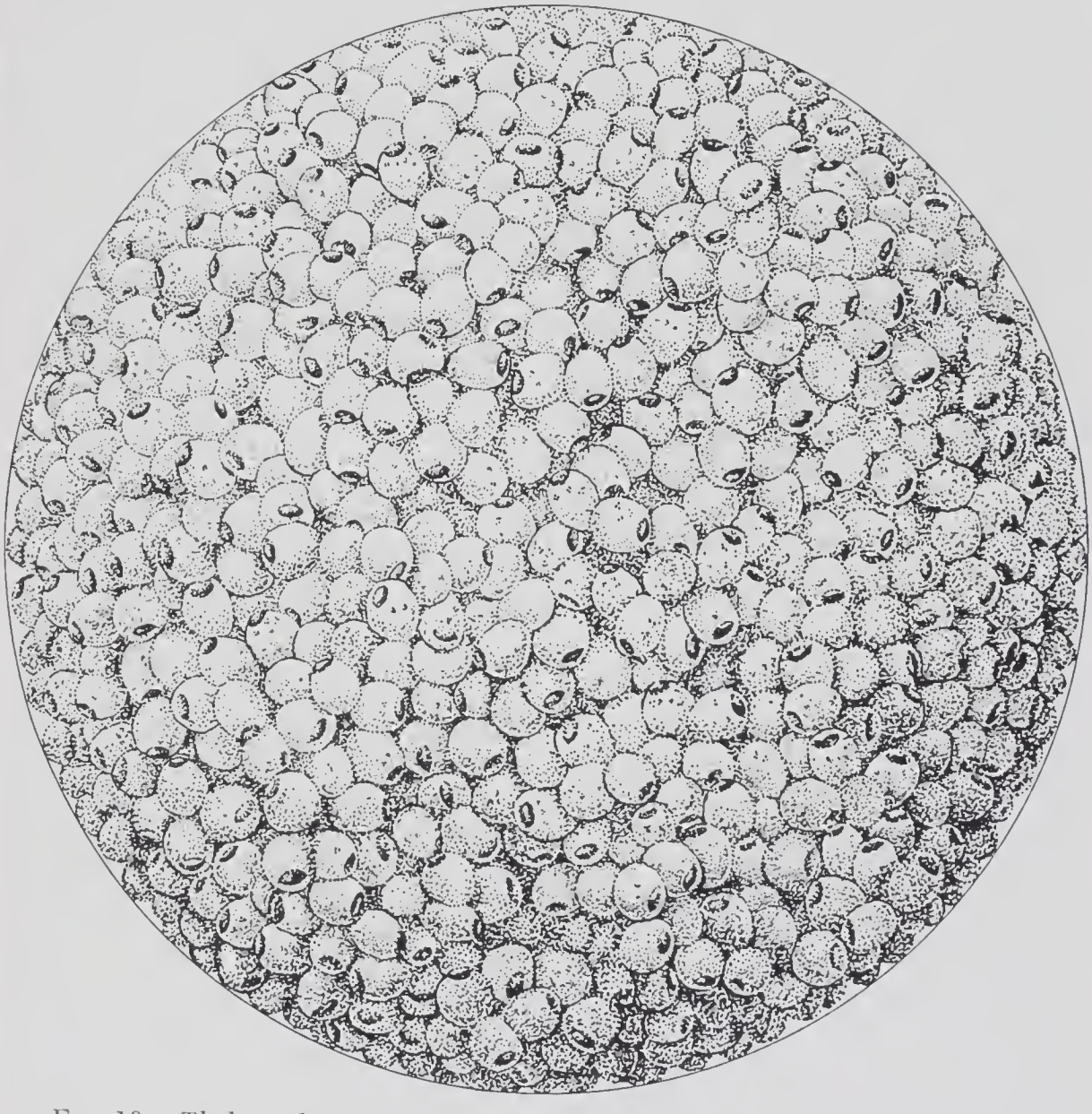
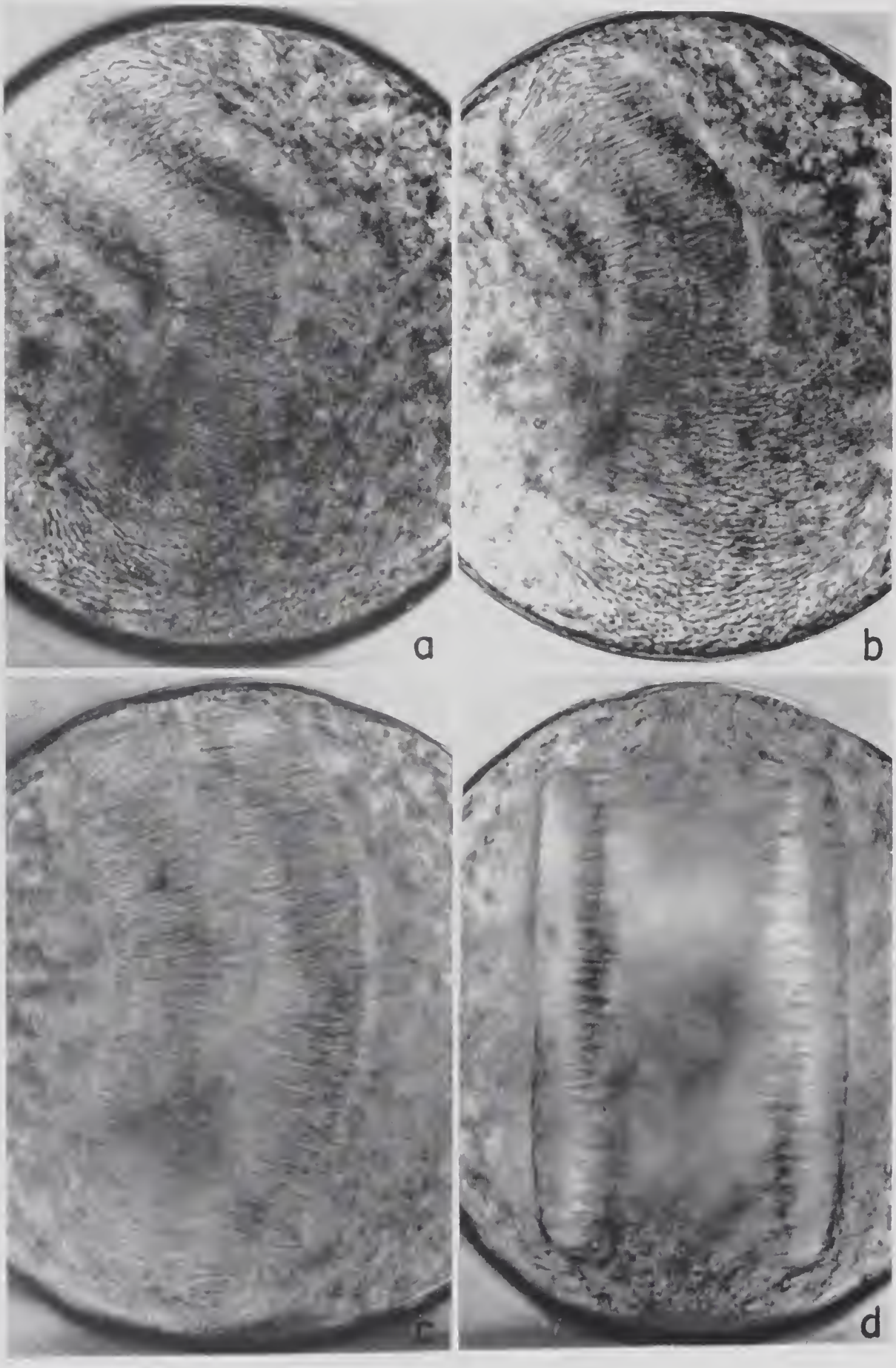


FIG. 10. *Thalassophysa sanguinolenta* (radiolarian). Central capsule in isospore formation. After Hollande and Enjumet (1953).

radiolarian developmental cycle. However, so far nobody has been able to observe the further development of these swarmers and their growth leading to the final stages which appear in the plankton. It is believed that this development takes place in the depths of the sea.

The following descriptions are confined to types whose cytology has been thoroughly investigated.


a
b
c
d

1. Species of *Thalassicolla* belong to the suborder Peripylea (Collodaria). In this genus we find some of the largest radiolarians of the marine plankton. The nuclei were first studied in detail by Huth (1913), who distinguished between two different lines along which development to swarmers could proceed: a "*Schlauchkernserie*" (= anisosporogenesis) and a "*Spindelkernserie*" (= isosporogenesis). The first of these series needs no further discussion: as indicated above, we are dealing with a parasitic dinoflagellate which develops first within the nucleus of *Thalassicolla* and breaks subsequently through the nuclear envelope with tubular protuberances to infest the cytoplasm.

The processes leading to isosporogenesis have recently been investigated by Hollande and Cachon-Enjumet (1959). They consider the nucleus of *Thalassicolla*, in agreement with Hartmann (1909, 1952), as "polyenergid." The nucleus which measures more than 300 μ in diameter in fully grown specimens is thought to acquire its full size by endomitotic polyploidization in the course of development from the swarmer to the final stage. Prior to formation of the next generation of swarmers, this "primary" nucleus disintegrates to form numerous "secondary" nuclei which become the nuclei of the isospores. Figure 10 shows isosporogenesis in a related form, *Thalassophysa sanguinolenta*.

To the best of our present knowledge, swarmer formation is the only mode of reproduction in *Thalassicolla*. Binary division of the cell or the nucleus has not been observed thus far and probably never does take place.

2. In *Aulacantha scolymantha*, which belongs to the suborder Tripylea, the situation is different. The cytology of this form has been studied mainly by Borgert (1900, 1909) and Grell (1953c). Borgert described anisosporogenesis as well as isosporogenesis in this species.

In contrast to *Thalassicolla*, the nucleus of *Aulacantha* divides regularly by binary fission. Aside from the unusual number of chromosomes (more than 1500), which is higher than in any other organism, this nuclear division shows certain peculiarities which distinguish it from the usual type of mitosis (Fig. 11). In the first place, the chromosomes form a contorted plate which occupies the whole width of the central capsule. Within the plate, the chromosomes are densely packed and

FIG. 11. *Aulacantha scolymantha* (radiolarian). Central capsule, stages of nuclear division in life: a, b, from the same specimen; c, d from different specimens. Notice the contorted shape of the mother plate in a. During separation of the chromosome masses, the daughter plates straighten out (b–d). After Grell (1953c).

wound about each other while their projecting ends are frequently bent like walking-canes. Chromosomal doubling occurs at this stage. The whole "mother plate," which shows but little similarity with an equatorial plate, proceeds then to divide into two daughter plates. From observations of the living cell, one gets the impression that both chromatids stay on the same side of the divisional figure and would thus be included in the same nucleus rather than being relegated to different daughter nuclei. Although the cytoplasm between the receding daughter plates appears lighter and more homogeneous than outside the divisional figure,

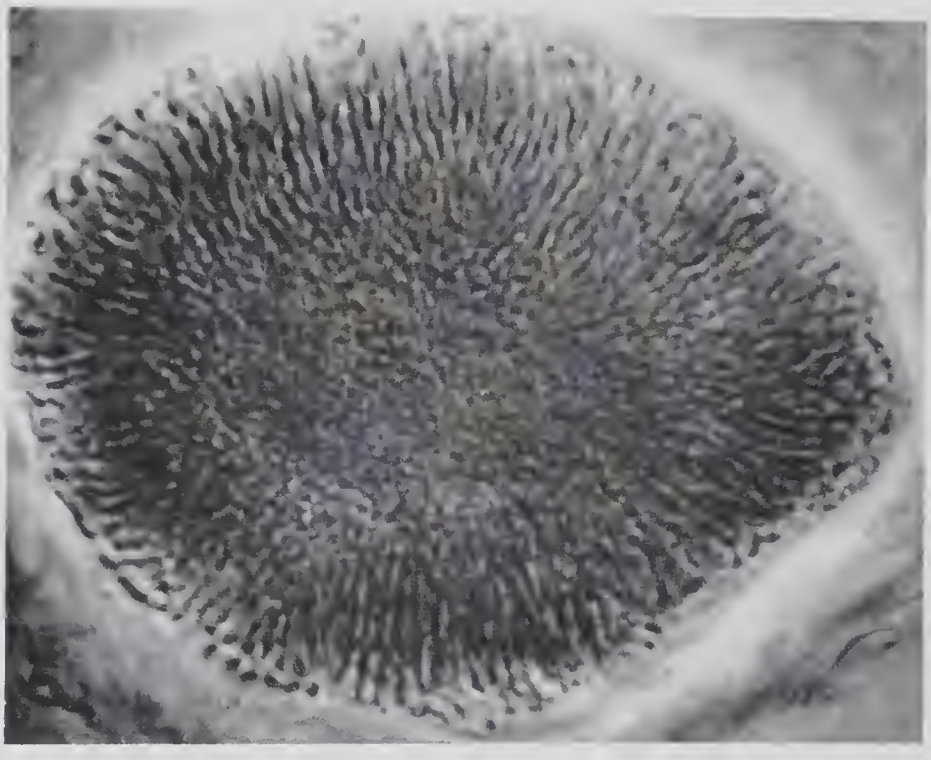


FIG. 12. *Aulacantha scolymantha.* Isolated daughter nucleus, Feulgen. Magnification: × 1200. After Grell (1953c).

there is neither an indication of the existence of a spindle apparatus, nor are there recognizable centrioles. Their occurrence seems rather improbable if only for the fact that, at the start of their recession, the daughter plates are still contorted. It is only later on that they assume the shape of plane-parallel disks. During the reconstruction of daughter nuclei, the margins of the disks turn up. Hence, these chromosome ends which were pointing toward the divisional figure come to be directed toward the outside. After reconstruction, the daughter nuclei show therefore a radial structure (Fig. 12). Major portions of the process of division were recently filmed (Grell, Film C 829).

In most specimens of *Aulacantha scolymantha* from the surface plankton, the nuclei contain a homogeneous but radially fissured, inten-

sely Feulgen-positive mass. These will be called "resting nuclei" (Fig. 13).

Besides specimens with resting nuclei, one finds individuals with clearly distinguishable chromosomes in the nucleus (Fig. 14). Borgert considered these as stages of prophase. However suggestive, there are

FIG. 13. *Aulacantha scolymantha.* Isolated central capsule with "resting nucleus." Astropyle on top with extruded cytoplasm. After Grell (1953c).

two peculiarities which make this interpretation seem questionable. (1) They show all phases of longitudinal splitting of chromosomes. However, this reduplication is different from the one observed in mother plates

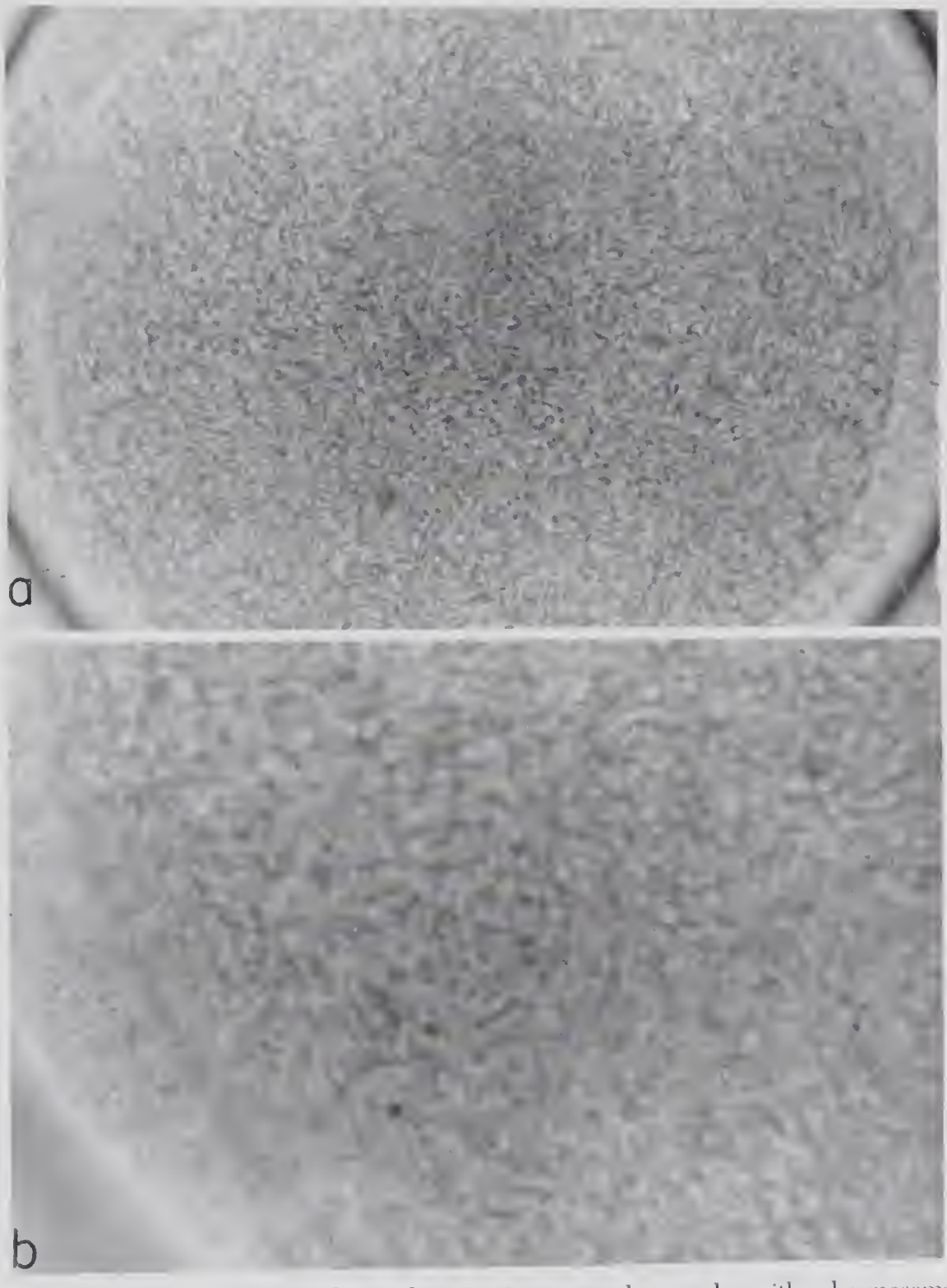


FIG. 14. *Aulacantha scolymantha.* Living central capsule with chromosomal masses in the nucleus. b. Portion of (a) at higher magnification. Duplication of the chromosomes is recognizable. After Grell (1953c).

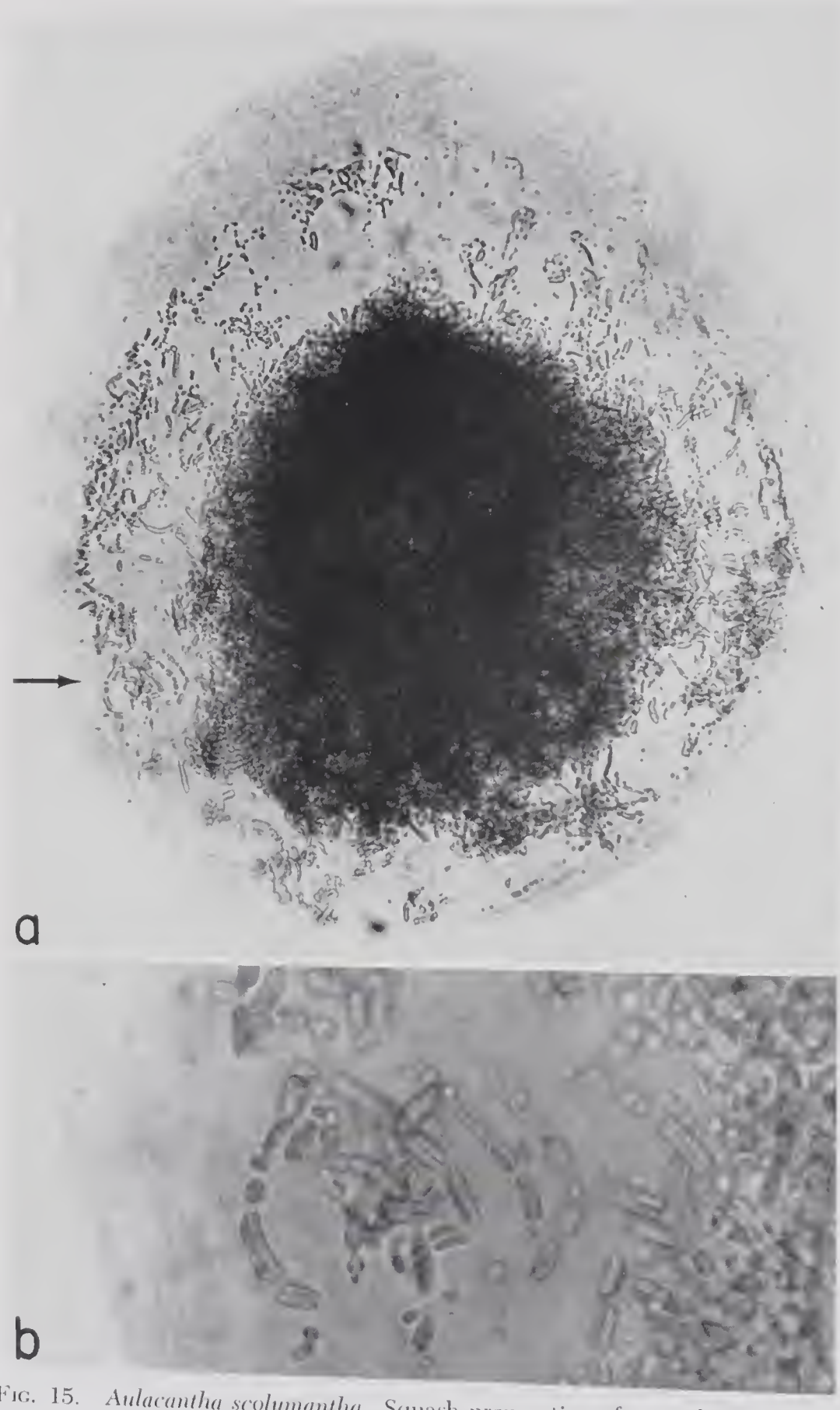


FIG. 15. *Aulacantha scolymantha*. Squash preparation of central capsule, stained with acetocarmine. The arrow points at magnified portion shown in (b). After Grell (1953c).

as it is connected with a whole cycle of chromosomal coiling of its own. (2) When the chromosomes are maximally condensed, a clear-cut longitudinal segmentation is recognizable in them (Fig. 15).

Since nuclei of the same structure, but much smaller over-all size, can be found, Grell believed he was dealing with stages of endomitotic polyploidization. The segmentation might mean that the chromosomes of *Aulacantha scolymantha* are in reality composite structures ("*Sammelchromosomen*"), each comprising a whole genome. The peculiarities of nuclear division in this species could thus be interpreted on the grounds that all chromosomes are genetically homologous. The decision on the hypothesis that the nucleus of *Aulacantha* is endopolyploid will depend upon whether or not multiple division of the "primary nucleus" as described by Borgert does indeed take place. Neither Hollande and Enjumet nor Grell (unpublished) have so far been able to find clear indications of this.

Hence, the chromosomes of *Aulacantha* continue to be a riddle urgently demanding its solution.[4]

B. Meiosis

Since meiosis is a nuclear event intimately connected with sexual reproduction, it is not of universal occurrence in protozoa. It can be interposed in protozoan life cycles (Fig. 16) in three different ways. In haploid protozoans (phytomonadina, some polymastigotes such as *Trichonympha*, *Eucomonympha*, *Barbulanympha*, *Oxymonas*, *Leptospironympha*, and *Saccinobaculus*, and the gregarines and coccidians among the sporozoans), meiosis takes place when the zygote divides (zygotic meiosis). In diploid organisms, meiosis immediately precedes the formation of gametes (gametic meiosis). This is the case in some polymastigotes (*Notila*, *Urinympha*, *Rhynchonympha*, and *Macrospi-*

[4] The endopolyploidy hypothesis has recently been totally rejected by Cachon-Enjumet (1961). She considers the division of the *Aulacantha* nucleus a true mitosis. It is not possible to enter into the details of her arguments at this point. I should like to remark, however, that her figures of supposed spindle fiber attachments (her Plates IV i and VIII i) seem to me only bends or breaks between adjacent units of the segmented *Sammelchromosom*. The fact that the ends of some chromosomes project from the bulk of the receding daughter plates is in itself not sufficient proof of real differences in chromosome length. Stickiness of some chromosomes during the separation of daughter plates might explain this observation. Finally it should be pointed out that the homology of all chromosomes in the nucleus of *Aulacantha* and the nonmitotic character of its division can be considered independently of the possibility of endopolyploidization. Even if the high number of chromosomes is not the result of endomitoses during individual development, homology of all elements is not excluded.

ronympha), some Heliozoa (*Actinophrys* and *Actinosphaerium*), and in ciliates. If a diploid, asexually reproducing generation ("agamont") alternates with a haploid, sexually reproducing generation ("gamont"), meiosis takes place between both generations at those nuclear divisions which precede the formation of gamonts (intermediary meiosis). This type of "heterophasic" alternation of generations is of widespread occurrence in the plant kingdom; in animals it is restricted to Foraminifera.

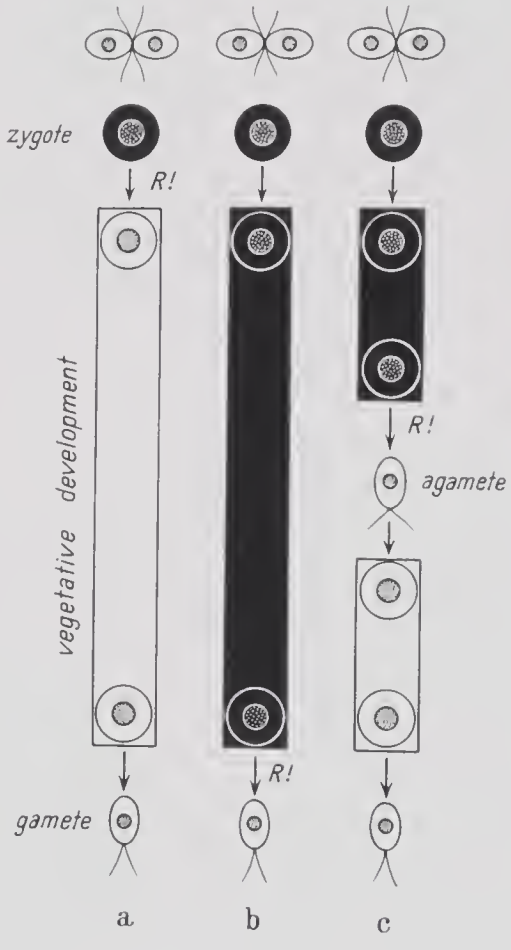


FIG. 16. Diagram of the alternation of haploid (white) and diploid (black) stages in the life cycles of protozoa. The position of meiosis is indicated by *R!*. a. Haploid form with zygotic meiosis. b. Diploid form with gametic meiosis. c. "Heterophasic" alternation of generations with intermediary meiosis. After Grell (1956).

As in other organisms, meiosis in most protozoans includes two nuclear divisions. In two-division meiosis, functional duplication of kinetochores is suppressed in the first division, while reduplication of the chromosomal arms is prevented in the second division. In these cases, the behavior of the chromosomes is not fundamentally different from that in multicellular organisms with localized kinetochores (see Rhoades, in Volume III of this treatise). Yet there are differences in

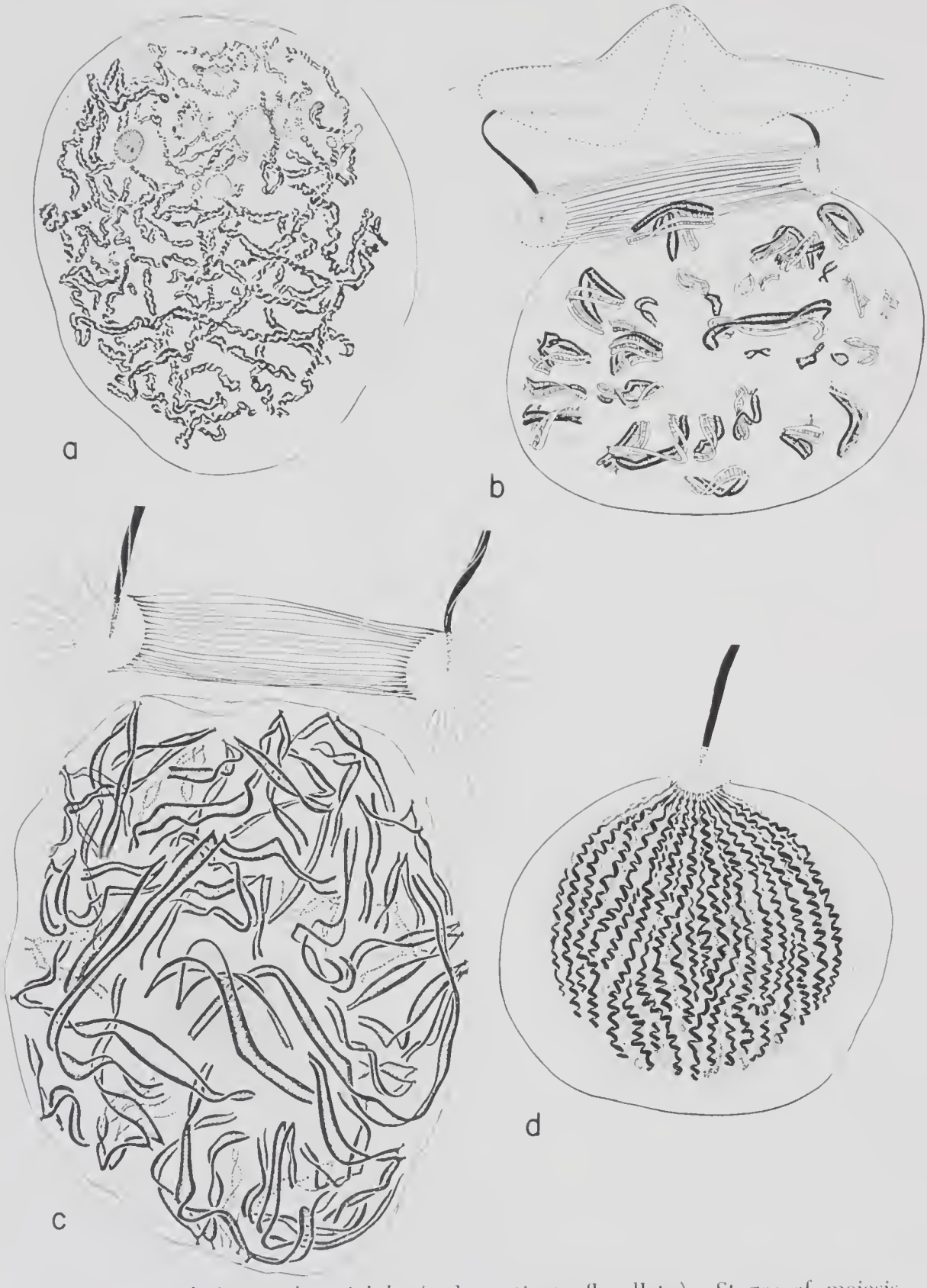


FIG. 17. *Barbulanympha ufalula* (polymastigote flagellate). Stages of meiosis. a. Mid prophase; chromosomes reduplicated, chromatids showing relational coiling. b. Late prophase; formation of tetrads, pairs of homologs shown in black and white. c. Prophase II; each chromatid of a dyad with its own kinetochore. d. Telophase II. After Cleveland (1954c).

the timing of chromosomal reduplication as well as in the coiling cycle and other details. As examples we shall discuss three special cases with very favorable chromosomes. At the same time, these cases provide examples for zygotic, gametic, and intermediary meiosis, respectively.

In the polymastigote flagellate *Barbulanympha ufalula* (Cleveland, 1954c) (Figs. 17 and 18), whose extranuclear centrioles were discussed above (page 12), the reduplication of homologous chromosomes to chromatids occurs remarkably early. In synapsis pairs of rather condensed chromatids unite with each other. In this case, the tetrads are held together through pairing forces arising, in part, between sister chromatids ("sister strand pairing") and, in part, between nonsister

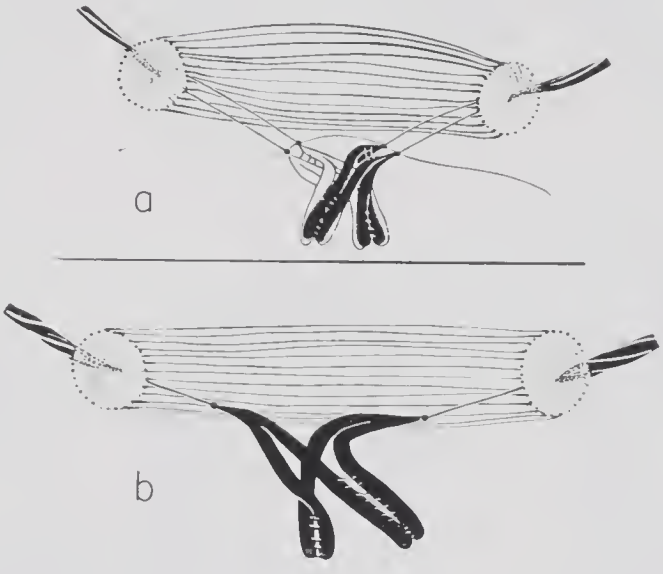


FIG. 18. *Barbulanympha ufalula*. Orientation of (a) a single tetrad at metaphase I and (b) a single dyad at metaphase II. After Cleveland (1954c).

chromatids ("nonsister strand pairing") rather than through chiasmata.[5] Although each of the chromatids has its own spindle attachment, the kinetochores of both sister chromatids behave as physiological units and become attached to the same spindle pole. Many details of the meiosis of *Barbulanympha* can be observed in life.

In the heliozoan, *Actinophrys sol*, the centrioles are replaced by polar caps and the chromosomes are arranged in a typical bouquet at the beginning of meiosis (Belar, 1922) (Fig. 19). At this stage, the polar cap is still single and the ends of the chromosomes are oriented toward it. Later on, chiasma-like configurations are observed. The dyads assume characteristic cross-forms at interkinesis owing to spread-

[5] We should like to draw attention to Cleveland's (1953b, 1954c, 1961) view on the origin of crossing over, which can, however, not be discussed here.

ing of the chromosomal arms and the presence of still undivided kinetochores.

Unlike *Actinophrys sol* and the monothalamic foraminiferans, such as *Myxotheca arenilega* (Grell, 1956) and *Allogromia laticollaris*

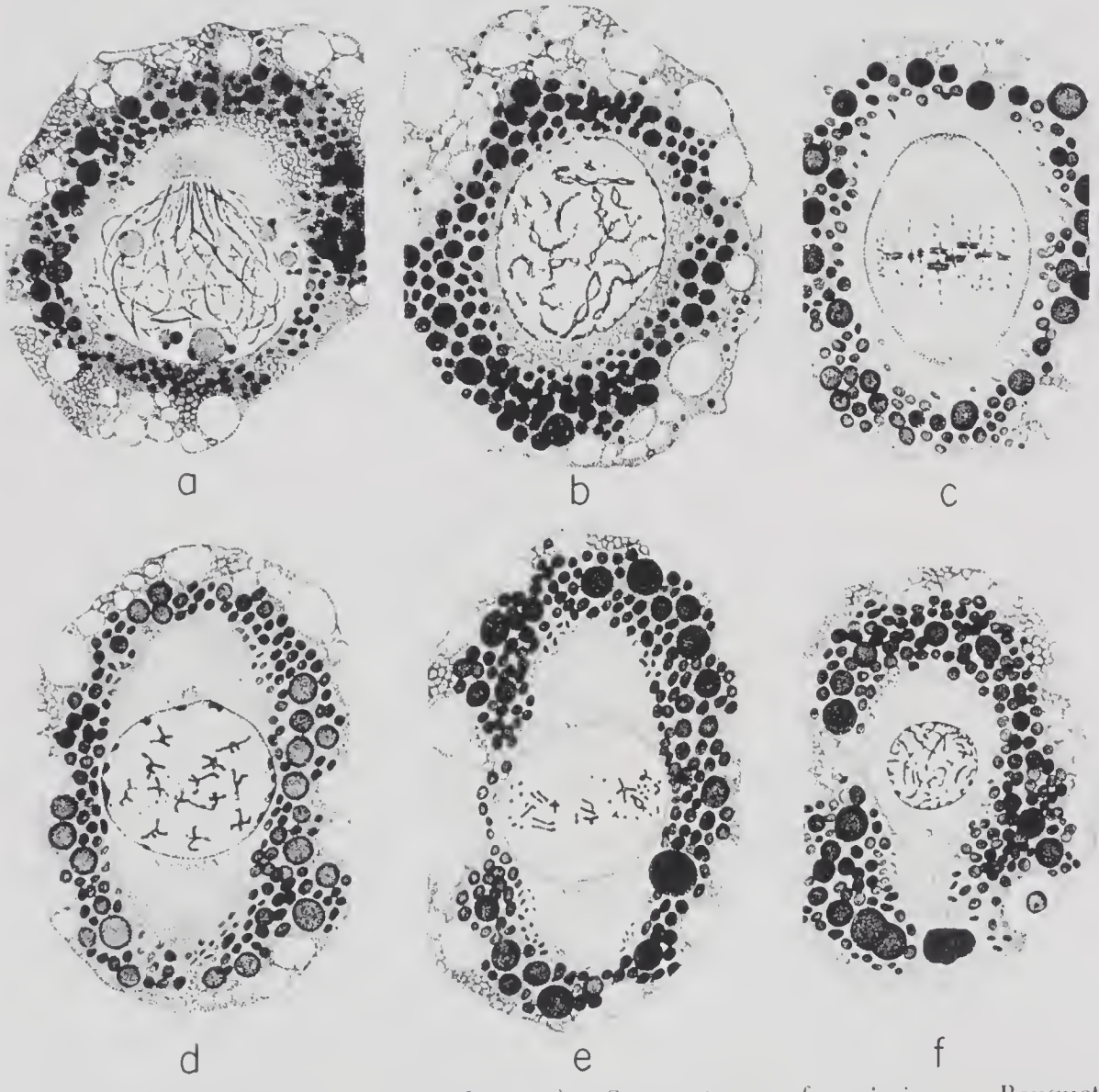


Fig. 19. *Actinophrys sol* (heliozoan). Some stages of meiosis. a. Bouquet stage. b. Strepsinema, homologous chromosomes apparently twisted around each other. c. Metaphase I. d. Interkinesis. e. Metaphase II. f. Gamete nucleus, pycnotic daughter nuclei at lower portion of figure. Magnification: × 1950. After Belar (1922).

(Arnold, 1955), the onset of meiosis in *Patellina corrugata* (Grell, 1959) (Figs. 20 and 21), which has intranuclear centrioles like all foraminiferans, is not characterized by a chromosomal bouquet. Instead, the chromosomes of this species appear irregularly entwined within the nucleus. At a later stage of prophase, which may be designated as

diplonema, the homologs are relationally twisted about each other. It cannot be decided whether or not chiasmata are formed between them. If so, they should be completely terminalized by the time diakinesis commences, as the bivalents are connected only by their ends at that stage. At metaphase, the coorientation of the bivalents, some of which

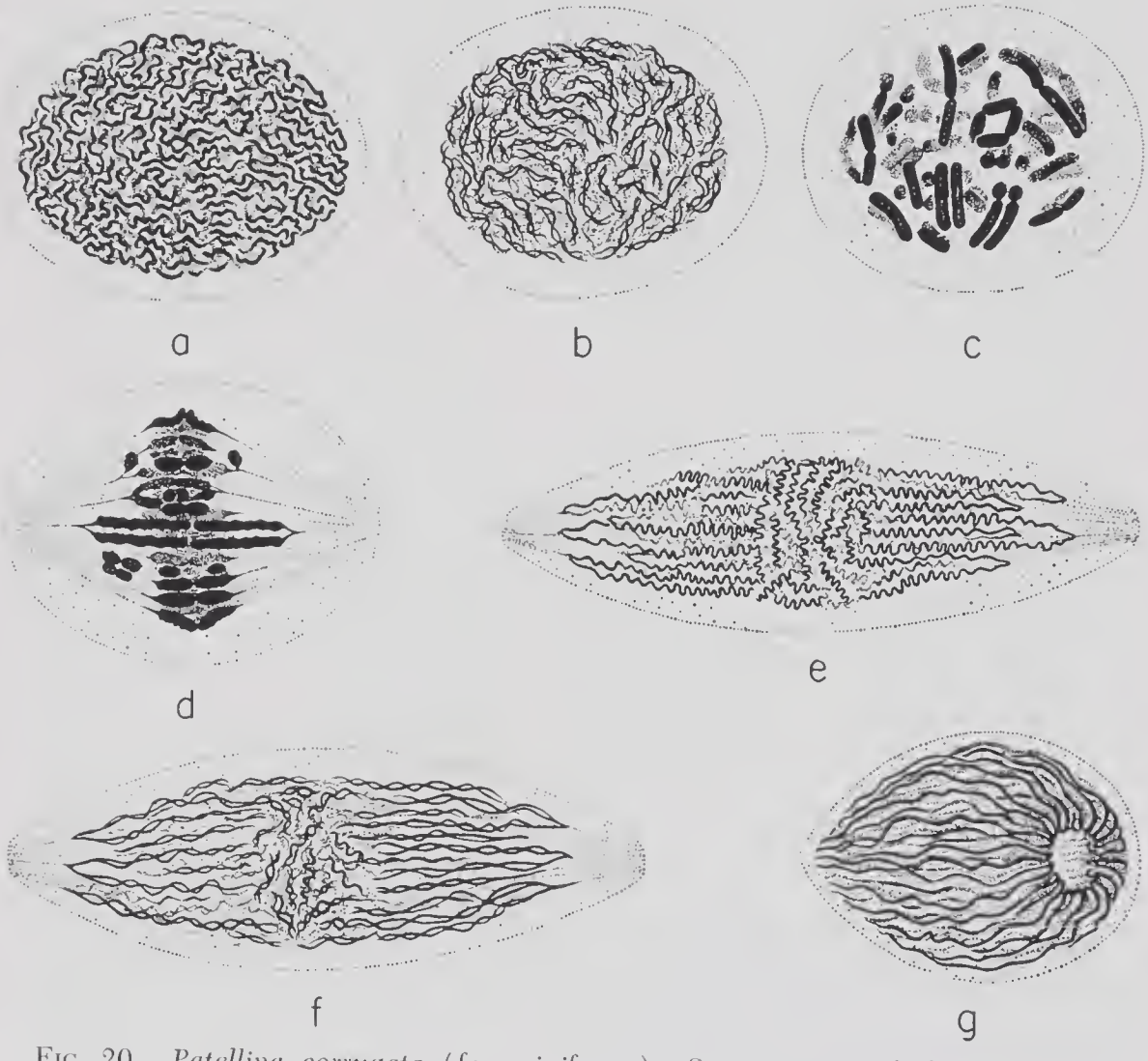


FIG. 20. *Patellina corrugata* (foraminiferan). Some stages of the first meiotic division. Nucleoli are not shown. a. Early prophase. b. Mid prophase. c. Late prophase (diakinesis). d. Metaphase I. e, f. Anaphase. g. Telophase. Feulgen preparations. After Grell (1959).

are of rather different sizes, is clearly recognizable. In the dyads, uncoiling occurs in step with the progression of anaphase movement; this particular feature is really quite striking (see Fig. 21).

In other foraminiferans, the course of meiosis is presumably much like that in *Patellina corrugata*. The peculiar behavior of the somatic nucleus in heterokaryotic species during meiosis of the generative nuclei will be discussed in the next section (see page 36).

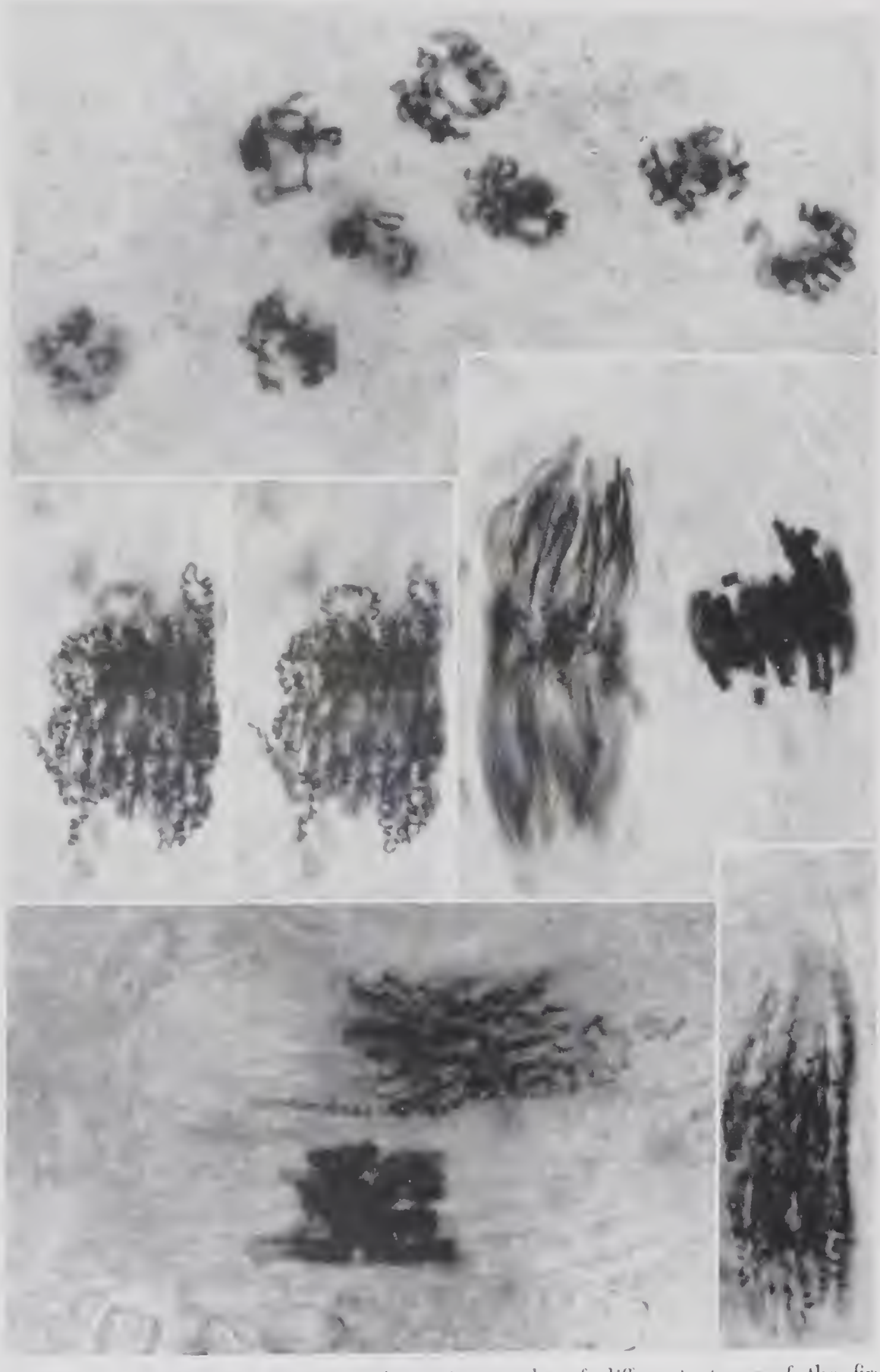


FIG. 21. *Patellina corrugata.* Photomicrographs of different stages of the first meiotic division (compare with diagrams Fig. 20). After Grell (1959).

Although in Protozoa meiosis generally involves two divisions, there are cases in which it occurs in one division (Cleveland, 1947, 1950a,b,c, 1951a,b). In such one-division meioses, the duplication of kinetochores as well as chromosomes is suppressed in prophase. In contrast to two-division meiosis, there are, of course, no tetrads and no chiasmata; however, this does not exclude the pairing of homologs and their coorientation at metaphase. In polymastigotes living in the gut of the North American roach *Cryptocercus punctulatus*, Cleveland found one-division meioses in the haploid genera *Leptospironympha*, *Oxymonas*, and *Saccinobaculus* (zygotic one-division meiosis) as well as in the diploids *Urinympha* and *Notila* (gametic one-division meiosis).[6]

Some observations indicate that one-division meiosis may also occur in Sporozoa; however, too little is known about the chromosomal events in this group to make this statement with absolute certainty.

IV. Nuclear Differentiation

Under this heading we include all cases where differences exist between cell nuclei although their gene contents are alike. Nuclear differentiation may lead to the realization of different cellular properties which can be demonstrated by suitable methods. For multicellular organisms, Briggs and King (see Volume I of this treatise), using nuclear transplantation as an experimental approach, were the first to show that cell nuclei become functionally different from a certain stage of development on. In Protozoa, it has long been known that genetically identical nuclei can call forth different cellular traits (Sonneborn, 1960). Since there are no recognizable structural differences between these nuclei, we may call this *functional differentiation.*

The following section will be devoted to differences in structure between nuclei of the same genetic constitution. *Structural differentiation* of nuclei is easily recognizable and so familiar that we tend to overlook the problems involved, and yet it is surely not of less importance than functional differentiation. There is no reason to suppose that both types are fundamentally different; instead, both seem to be ex-

[6] Although, in the case of haploids, it might be argued that the second division has been overlooked, this is quite impossible in diploid forms. In these the developmental cycle would become unintelligible if meiosis were not to occur in a single division. Much as we may be unaccustomed to the idea of meiosis in one single division, there is no good reason to doubt its existence. The history of research on meiosis shows how often dogmatic concepts have hindered understanding of these processes. It seems we have to put up with the fact that nature loves variability more than we do.

pressions of the same property of the genetic material, namely, its capacity of *differential gene activation.*

In multicellular organisms, structural differentiation of nuclei is clearly connected with somatic differentiation. Thus, different cell types of the body may be characterized by differences in nuclear structure. The latter may depend upon the number of chromosome sets (endopolyploidy), size of the chromosomes, kind of heterochromasy, and amount of nucleolar and nuclear ground substances.

Obviously, there is no somatic differentiation in unicellular organisms (see Grell, 1956). Yet there are states of structural nuclear differentiation which can either successively or simultaneously be attained.

A. Successive Nuclear Differentiation

Although somatic differentiation is absent in protozoa, different cell types with characteristic differences in nuclear structure can appear in the course of the same life cycle. This is most striking in Protozoa with a regular alternation of generations, as in Foraminifera (Fig. 23) and Sporozoa (Fig. 39). The names of successive stages (gamonts, gametes, zygotes, agamonts, schizonts, merozoites, sporozoites, etc.) indicate that we are dealing with a sequence of well-differentiated cells. In many cases these stages show specific differences in nuclear structure. As a rule, the nuclei of rapidly growing cells are large and rich in nuclear ground substance and nucleolar mass (for example: gamonts of Foraminifera, Eugregarinida, Eucoccidia) whereas nongrowing stages, such as gametes, are characterized by small nuclei consisting largely of chromosomal material.

Occasionally there is an abrupt transition from one nuclear type to the other, for example in *Myxotheca arenilega*, which was discussed above (Fig. 6).

As any process of differentiation, this type of nuclear differentiation raises two questions. (1) What causes the change from one nuclear type to the other? (2) Is the change reversible or irreversible?

With respect to the first question, we have reasons to assume that differentiation is not due to autonomous changes of the cell nuclei, but is induced through conditions arising within the cytoplasm in connection with the developmental cycle.

The second question might seem to be already answered by the mere fact that different nuclear structures appear in sequence as stages of the developmental cycle. While the progression from one step to the next is unidirectional, the cycle as a whole is repeated. Although stage

to stage reversibility may not exist, the process as a whole is obviously reversible. Is the structure of the nucleus at a given stage of the life cycle merely the outcome of the cytoplasm in which it finds itself, or is it necessary, for any nuclear structure to appear, that preceding nuclear stages have been run through? An answer to this question can be reached only through transplantations of nuclei between cells of the same species at different stages in the life cycle.

B. Simultaneous Nuclear Differentiation

Although there are many multinuclear protozoans (calonymphidae, opalinids, many amebae, heliozoans, radiolarians, stages of foraminiferans, and sporozoans), differentiation between the nuclei of the same cell occurs only in two groups, viz. some foraminiferans and most ciliates. It is practically certain that nuclear differentiation evolved independently in these two systematic groups, but there are nevertheless certain similarities in the general aspects of this phenomenon. In both cases we are dealing with nuclear dimorphism, and in both, this dimorphism is the expression of a differentiation in "*Keimbahn*" (germ line) and soma: the nuclei of one type (generative nuclei) are capable of unlimited division, whereas those of the second type (somatic nuclei) show either limited capacity to divide or are wholly incapable of it. Hence, they will degenerate sooner or later and have to be periodically reconstructed from descendants of the generative nuclei.

In contrast to the genuine nuclear dimorphism of foraminiferans and ciliates are cases with differently structured nuclei in the same cell which are, however, not of common descent. One example of this is found in amebae of the family Paramoebidae. Schaudinn (1896) found that *Paramoeba eilhardi* (Fig. 22), besides a "true" nucleus, has a so-called "*Nebenkörper*" (accessory body). Grell (1961) could show that the *Nebenkörper* is indeed a real nucleus. Although there are certain correlations in divisions of both nuclei, neither can give rise to the other type. Furthermore, there are considerable differences in DNA-content in both nuclei. On the basis of these facts, Grell considered that the *Nebenkörper* might be a parasitic nucleus. The "parasite" would be so well adapted to its host cell as to give up its cellular organization and, in a manner of speaking, become reduced to a nucleus. Although this interpretation is entirely hypothetical, it is quite certain that the nuclear situation in Paramoebidae is not equivalent to the type of nuclear dimorphism found in foraminiferans and ciliates. Incidentally, a similar hypothesis might be considered with respect to the much debated "kinetonucleus" of trypanosomes (kinetoplast of the Anglo-

American literature, blepharoplast of the German literature, see Mirsky and Osawa, Volume II of this treatise). As is well known, kinetoplasts contain DNA and might be thought to represent nuclei "degenerated" in connection with parasitism (Grell, 1962).

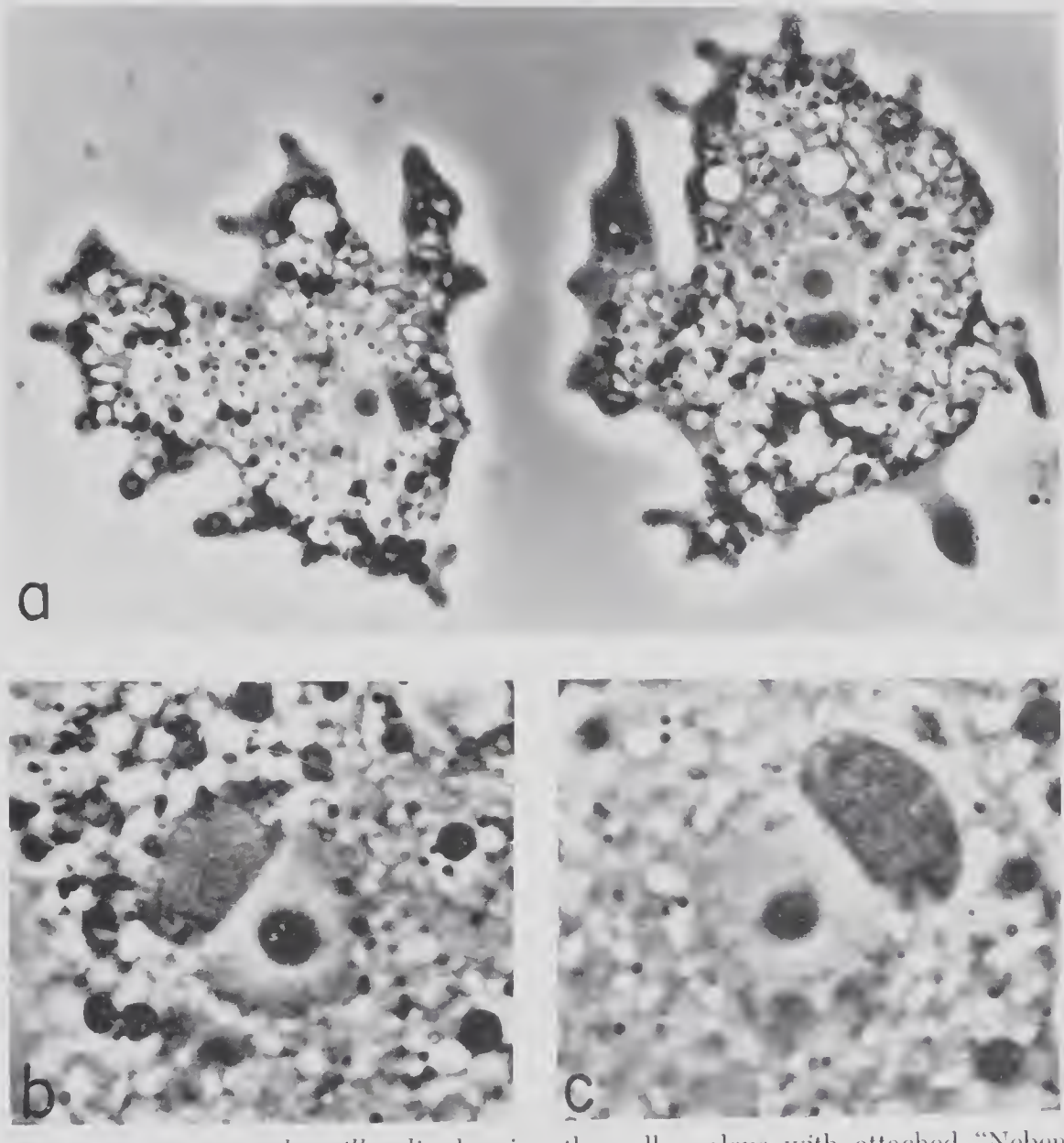


FIG. 22. *Paramoeba eilhardi*, showing the cell nucleus with attached "Nebenkörper." Living specimens, phase contrast. After Grell (1961).

1. Foraminifera

Although nuclear dimorphism in foraminiferans became known only a few years ago, we shall discuss it first because the cytological situation is in some ways simpler than in ciliates. Data on the development of foraminiferans are available for only a few species which could be cultured in the laboratory (Grell, 1954, 1957, 1958a, b, c, 1959). These

species belong to the Monothalamia (better "Archimonothalamia") and the Rotaliidae. Nuclear dimorphism has so far been found in only some of the Rotaliidae. Hence, we can distinguish between *homokaryotic* and *heterokaryotic* species. Only the latter will concern us here. As an example, *Rotaliella roscoffensis* (Fig. 23) will be discussed. As in most foraminiferans, development involves an alternation of two generations, both generations reproducing differently. The sexually reproducing generation is called the gamont; the asexually reproducing one, agamont. In *R. roscoffensis*, gamont and agamont can be distinguished by the sizes of their chambers. Cytological study shows that the number of nuclei is also different in both generations. The gamont has only one nucleus located in the initial chamber (proloculum). At the beginning of sexual reproduction (gamogony), the walls separating the chambers are dissolved and several nuclear divisions take place in the common cytoplasm. After the divisions, each gamete nucleus becomes surrounded with its own portion of cytoplasm. The gametes thus formed are amoeboid and copulate in pairs within the shell. Sexual reproduction, then, leads to autogamy. Within the zygote two nuclear divisions take place leading to agamonts with four nuclei. Later it may happen that one or two of the nuclei degenerate, leading to agamonts with three or only two nuclei. In any case—even if there are only two nuclei—nuclear dimorphism becomes established. One of the nuclei—but never more than one—will swell up, losing its compact structure, and form a nucleolus, while the others (3, 2, or 1, as the case may be) remain as condensed as before. The young agamonts emerge at this stage. After the formation of two or three chambers, the larger nucleus leaves the initial chamber and moves into one of the younger compartments while the smaller nuclei stay within the initial chamber. They will leave it only after the agamont has reached a certain critical size. With this, meiosis begins in which only the smaller nuclei participate while the larger nucleus degenerates. Therefore the small nuclei may be called generative while the larger one is to be considered the somatic nucleus. There are two meiotic divisions. Immediately thereafter, the agamont gives rise to a number of gamonts depending upon the number of generative nuclei previously present; thus, there are 12, 8, or only 4 gamonts if there were, before meiosis, 3, 2, or only 1 generative nuclei. As the size of the agamont is independent of the number of generative nuclei, the gamonts formed may be of different sizes.

Of cytological interest is the fact that the somatic nucleus, although incapable of actually completing meiosis, will to a certain degree "try" to participate in it. The decisive difference in the behavior of both nuclei

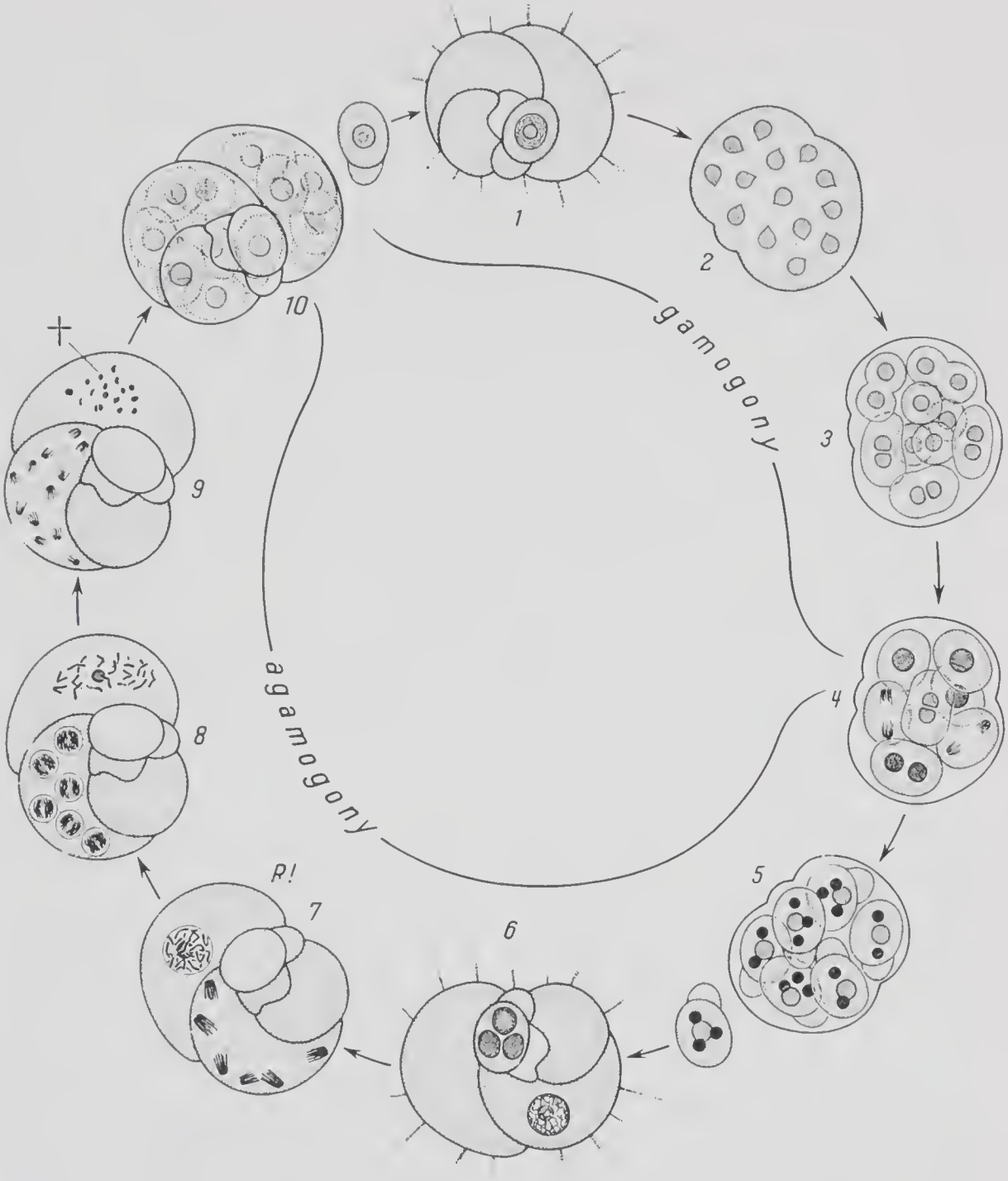


Fig. 23. *Rotaliella roscoffensis* (foraminiferan), developmental cycle. *1*. Gamont. *2*. Formation of gamete nuclei. *3*. Fusion of gametes in autogamy. *4*. Zygotes and first postzygotic division. *5*. Young agamonts, some containing three, some four nuclei. *6*. Fully grown agamont, generative nuclei in initial chamber slightly swollen indicating onset of meiosis, somatic nucleus in younger chamber. *7*. Anaphase of the first meiotic division, condensation of chromosomes beginning in the somatic nucleus. *8*. Metaphase II, chromosomes of somatic nucleus scattered in cytoplasm. *9*. Anaphase II, pycnosis of chromosomes of the somatic nucleus. *10*. Formation of gamonts. After Grell (1957).

is that homologous chromosomes will not pair in the somatic nucleus. They remain univalent ("asynapsis") but condense like normal meiotic chromosomes. There is even an intranuclear spindle; however, as is typieal for univalents, the ehromosomes cannot coorientate. After attachment to the spindle, they are distributed at random. The nuclear envelope dissolves before any daughter nuclei are formed. The chromosomes become pyenotic after getting into the cytoplasm and ultimately disappear.

The elimination of the somatic nuclei occurs in a similar manner in most heterokaryotie foraminiferans. One might think of elimination as a direct eonsequence of asynapsis at meiosis. However, this idea is refuted by the behavior of the somatic nucleus in *Rotaliella heterocaryotica,* the species in which nuclear dimorphism was first discovered. In this case, the only indication that the somatic nucleus participates in meiosis is its slight swelling. No spindle is formed. Right after swelling, the nucleus condenses again, becomes pycnotic, and disintegrates subsequently into a number of fragments which are dissolved in the cytoplasm. Although it is likely that pairing of homologs does not take place, lack of synapsis ean searcely be the cause of the elimination of the somatic nucleus.

A further differenee between *Rotaliella heterocaryotica* and *Rotaliella roscoffensis* is that after the postzygotic divisions, only the latter shows nuclear degeneration. Agamonts of *Rotaliella heterocaryotica* have therefore always three generative nuclei and one somatic nucleus.

Other hetcrokaryotie genera are different in starting sexual reproduction by pairing of gamonts (gamontogamy) and in peculiarities of nuclear dimorphism.

In *Rubratella intermedia* (Grell, 1958a), after two postzygotic nuclear divisions, one of the four daughter nuclei degenerates. The remaining three undergo a third division leading to the six nuclei of the young agamont. One of these is destined to become the somatic nucleus.

Glabratella sulcata (Grell, 1958b) diverges most from the *Rotaliella* pattern in that the number of postzygotic divisions is not strictly determined but varies within wide limits. Hence, the number of nuclei in emerging agamonts ean be very different (2–24), but only one of these nuclei is differentiated to a somatic nucleus. However, this is not the final state. During the growth phase of the agamont, further somatic nuclei are successively differentiated (Fig. 24). As soon as the first somatie nucleus has moved out of the initial chamber, another nucleus swells, forms nucleoli, and follows its predecessor. This may be repeated two or three times, leading to several somatic nuclei and a variable number of generative nuclei.

The phenomenon of nuclear dimorphism in Foraminifera poses a number of questions. It must remain open why dimorphism is found in some species only and, for that matter, why in only one of the two generations, namely the agamonts. The gamont, which is surely not less

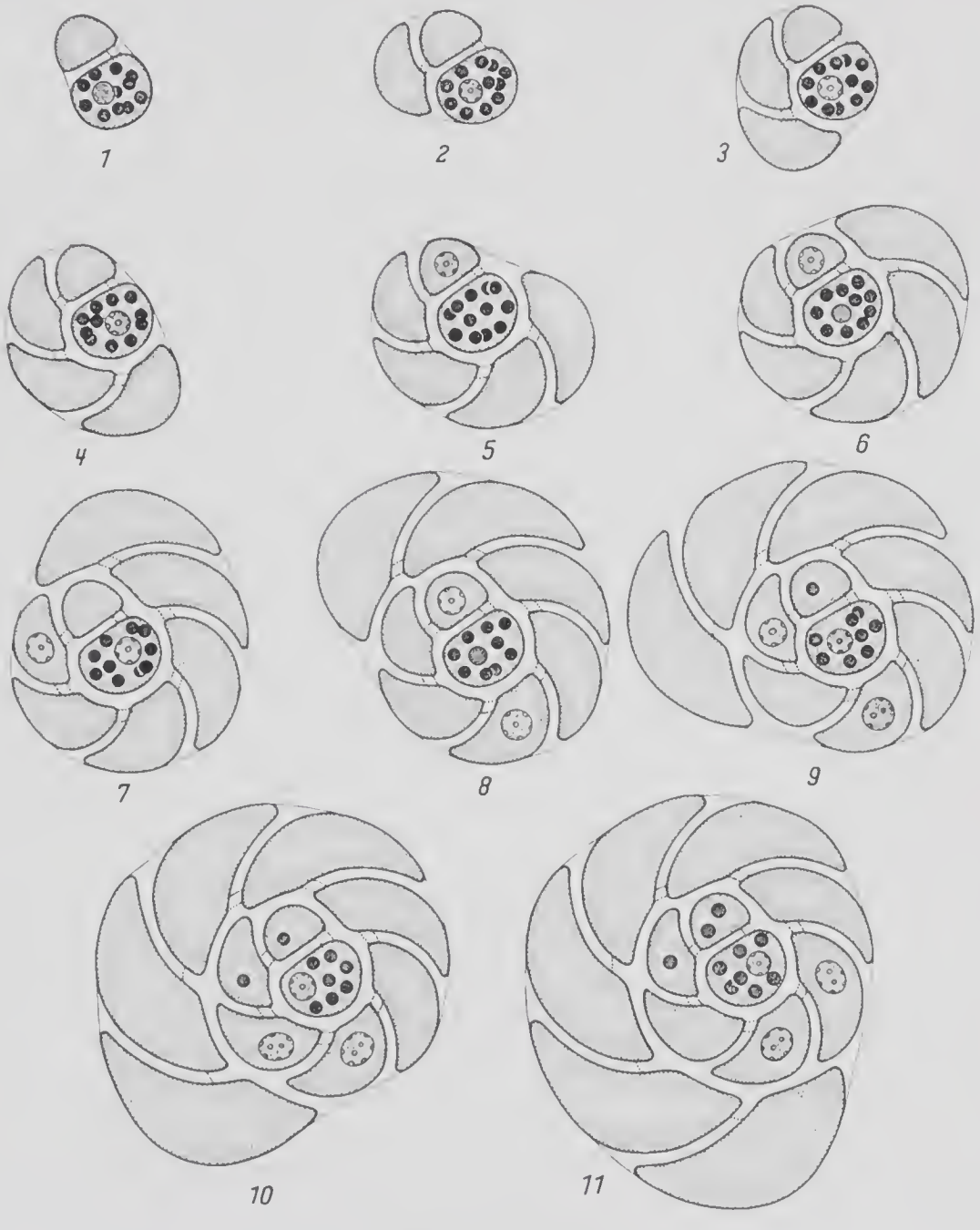


FIG. 24. See facing page for legend.

differentiated, needs only one nucleus—and a haploid one at that. What circumstances play a role in the agamont's need for two structurally different diploid nuclei?

While these questions must be left unanswered, the problem, whether

the existence of structural differentiation would also mean that both nuclei are functionally different can be answered quite clearly. The very fact that somatic nuclei are of less compact structure, have nucleoli, and move into regions of the cell where metabolic activities are probably greatest, indicates their preoccupation with cellular metabolism, whereas

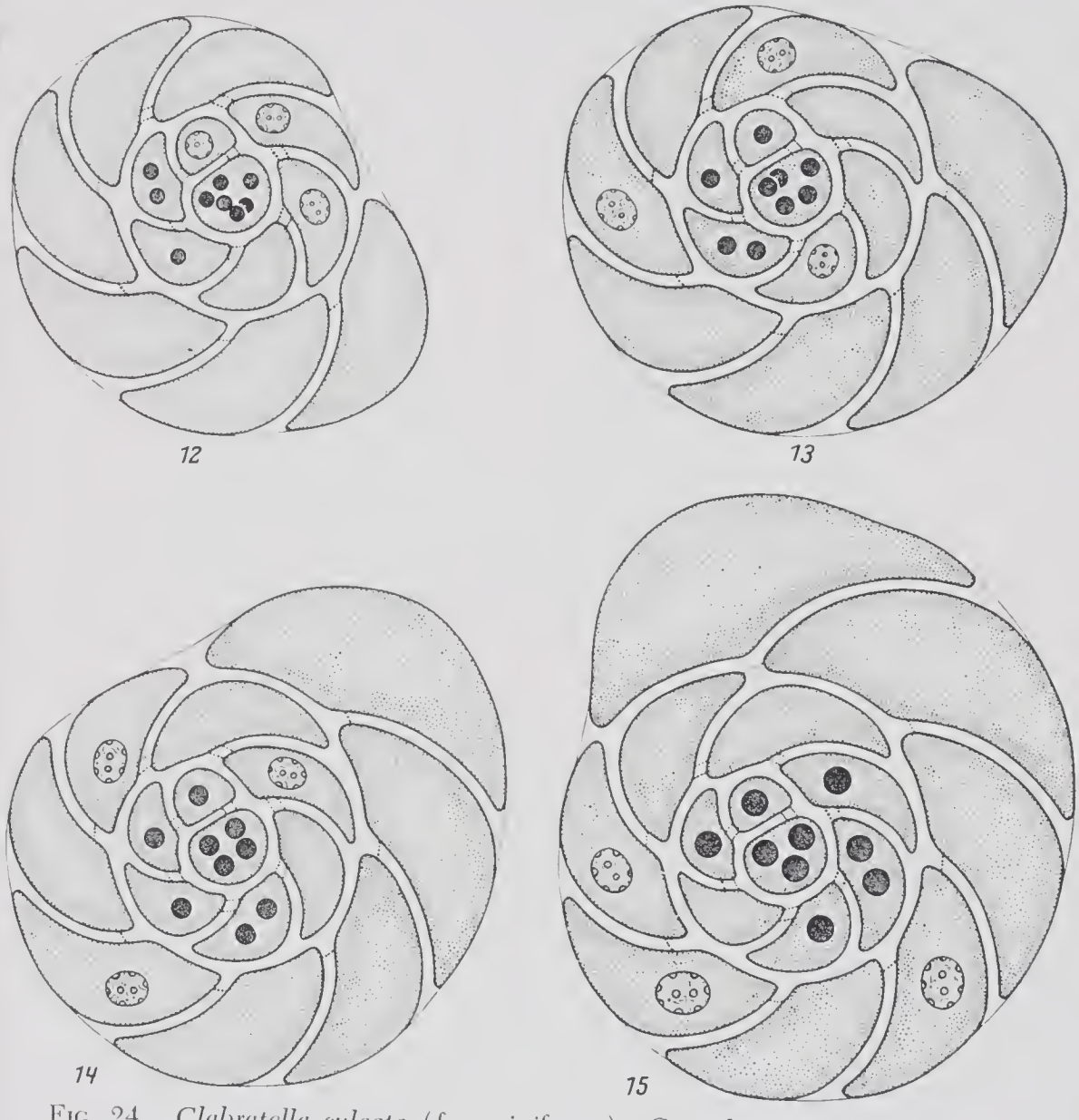


FIG. 24. *Glabratella sulcata* (foraminiferan). Growth stages of agamont, showing successive differentiation of somatic nuclei (see text). After Grell (1958b).

generative nuclei, on the basis of their density and their lack of nucleolar substance, seem less well equipped to handle physiological activities. This idea is supported by the data on *Rotaliella roscoffensis*. As discussed above, this species performs an interesting "experiment," inasmuch as some of its nuclei—for reasons unknown—degenerate after the second postzygotic division. Already the fact that agamonts may frequently

(22.5%) have only two nuclei and not rarely (1.4%) only one generative nucleus is most informative. However, the decisive point is that in exceptional cases all generative nuclei may be missing. Even these agamonts grow up normally. However, after reaching a certain size limit (which must therefore depend upon the somatic nucleus only), they die off. This is undoubtedly so because the somatic nucleus enters asynaptic pseudomeiosis according to its state of determination and becomes eliminated (Fig. 25).

Regarding our question on functional differentiation, we can conclude from this "Naturexperiment" (Boveri) that the somatic nucleus alone

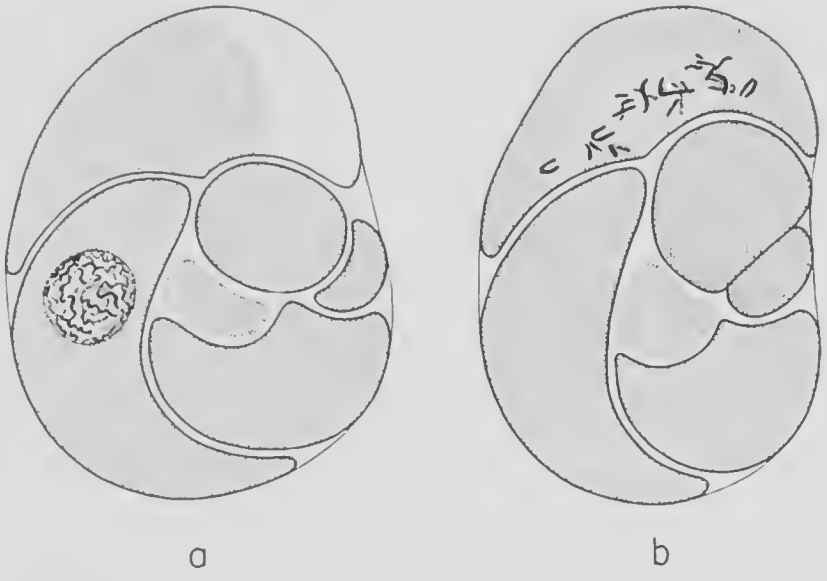


FIG. 25. *Rotaliella roscoffensis.* Abnormal agamont. Although no generative nuclei are present, the somatic nucleus undergoes asynaptic pseudomeiosis as normal, indicating cytoplasmic induction of this process. After Grell (1957).

is sufficient to support the growth of the cell. Evidently, it is capable of directing all metabolic activities connected with this. Hence, we may call it the *metabolic nucleus.* On the other hand, it is incapable of reproduction. This ability is restricted to the generative elements, which may thus be looked upon as the "*reproductive nuclei*" of the cell.

With respect to the problem of differentiation, the following question is of primary interest: what are the conditions leading to differentiation between both types of nuclei? Two observations, made with *Rotaliella roscoffensis,* were most elucidating in this context. (1) Degeneration of nuclei takes place right after the second postzygotic division, i.e., *before* any differentiation is recognizable. (2) In spite of this, one (but never more than one) of the remaining nuclei is always determined as a somatic nucleus.

These observations suggest that determination is not the result of any segregational mechanism connected with the postzygotic divisions. Instead, it must be due to the general condition of the cytoplasm which, in *Rotaliella roscoffensis*, determines that only one of the remaining nuclei embarks upon the road toward somatic differentiation.

This concept is further corroborated by *Glabratella sulcata*, where (as discussed above) several somatic nuclei are successively differentiated. Even if the agamont has only very few (for instance, two) nuclei, there is always one determined as a somatic nucleus right from the start (cf. Fig. 26a). When such a binucleated agamont reaches a certain size, the second nucleus also becomes somatic after some time (Fig.

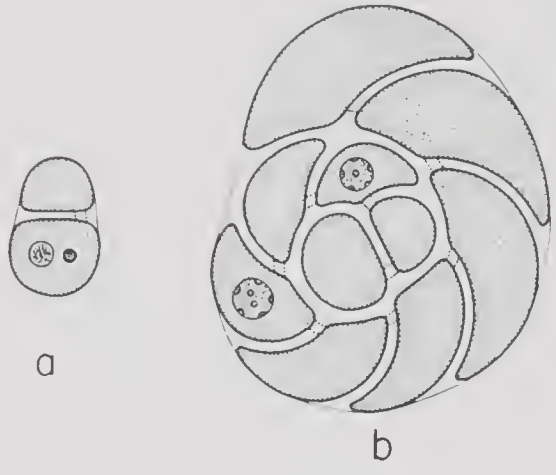


FIG. 26. *Glabratella sulcata*. Abnormal agamont with two nuclei. a. Young stage containing a generative and a somatic nucleus. b. Later stage, second nucleus also differentiated into a somatic one. After Grell (1958b).

26b). Assuming that determination is also irreversible in this case, the cell ought to suffer the same fate as the uninuclear agamonts of *Rotaliella roscoffensis*.

In Fig. 27 a hypothesis is outlined that may account for these relationships. There are two assumptions involved:

1. The cytoplasmic state is due to a determinative substance whose concentration increases with time (this situation is indicated by the sloping line).

2. The cell nuclei are different in their sensitivities toward this substance (this situation is indicated by drawing the nuclei on top of each other).

According to this hypothesis, the nuclei would "normally" (if there were no determinative condition) continue to be generative. Hence, there is only one direction which determination takes, namely, toward somatic nuclei, and the nucleus which is "most sensitive" will be the first

to react in the presence of the determinative substance. In *Rotaliella*, which has only four nuclei, and in *Rubratella* with six nuclei, only one somatic nucleus is differentiated. In *Glabratella* we may be dealing with a system of graded sensitivities leading to successive determination of several somatic nuclei.

Different subsidiary assumptions could be made to account for the fact that no further somatic nuclei are formed in *Rotaliella and Rubratella*. Thus, the production of determinative substance may cease, or a somatic nucleus, once formed, may inhibit the determination of other

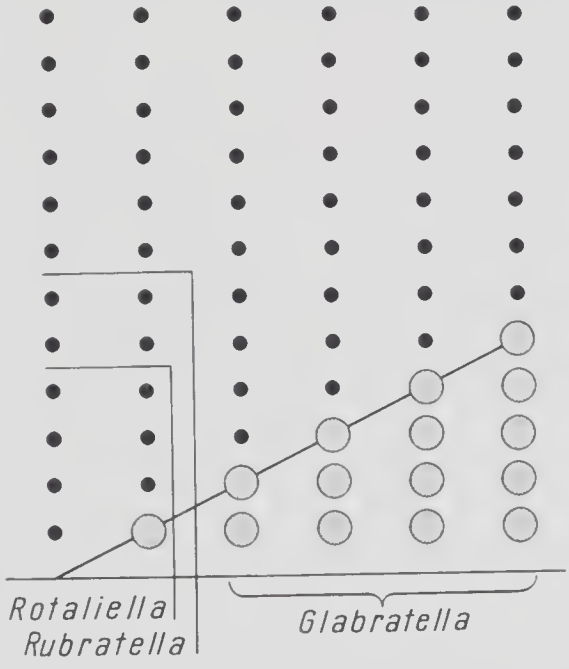


FIG. 27. Diagrammatic representation of the hypothesis outlined in text (page 43). After Grell (1958b).

nuclei. Recent experiments with *Rotaliella heterocaryotica* (Czihak and Grell, 1960) would seem to support the second alternative. In contrast to *Rotaliella roscoffensis*, there is no nuclear degeneration after the second postzygotic division in this species, and the agamonts therefore always have one somatic nucleus and three generative nuclei. In favorable cases the somatic nucleus is discernible in the living cell and can be inactivated by ultraviolet microbeam irradiation. Some time after ultraviolet inactivation, one of the generative nuclei within the initial chamber enlarges and acquires a nucleolus, thus changing into a new somatic nucleus. After meiosis such agamonts form only eight gamonts instead of twelve (Fig. 28).

This experiment does indeed demonstrate an inhibiting influence from the somatic nucleus; this influence can be removed by the inactivation of

the somatic nucleus. One could envisage a substance formed by the somatic nucleus destroying the determinator as soon as it is formed.

At any rate, we seem to have uncovered a feedback cycle of the type operating in determinative processes elsewhere. A beautiful example of this is furnished by studies on the developmental physiology of the lens in the vertebrate eye. After its removal a new lens is formed from the margin of the iris; a lens previously transplanted into the vitreous body, however, inhibits regeneration by the margin of the iris.

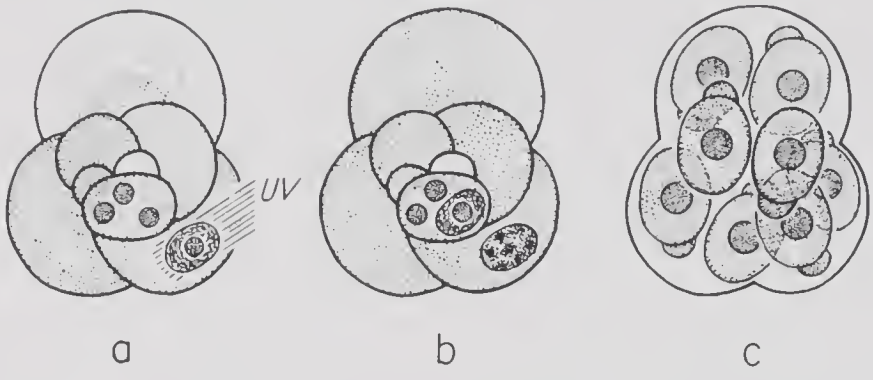


FIG. 28. *Rotaliella heterocaryotica.* Inactivation of the somatic nucleus of a living agamont by ultraviolet microbeam irradiation (a). After inactivation, one of the three generative nuclei in the initial chamber differentiates into a somatic nucleus (b). After meiosis eight gamonts are formed instead of twelve (c). After Czihak and Grell (1960).

These observations and experiments were treated in considerable detail, not only because of the present author's special interests, but also on the basis of his conviction that feedback processes of this kind play an important role in nucleocytoplasmic interrelations.

2. Ciliata

The discovery of nuclear dimorphism in foraminifera made the caryological situation in ciliates appear much less distinetive. Also the fact that electron microseopy does not reveal any prineipal difference between cilia and flagella has done much to diminish the apparent gap between the ciliates and the remainder of the protozoa. It seems unjustified to the present author to persist in separating the ciliates as "Cytoidea" from all other protozoa ("Cytomorpha").

Besides, recent investigations have shown that nuclear dimorphism as known from most ciliates is not as uniform a feature as was hitherto supposed. Already in 1923, Lwoff described a peculiar marine eiliate nnder the name *Stephanopogon mesnili* whieh has according to his investigations no nuclear dimorphism. Later on (1936), he presented this

in more detail. Other investigators (Fauré-Fremiet, 1954; Dragesco and Poljansky, personal communications) confirmed this point. Disregarding the opalinids (which are frequently adjoined to the flagellates on the basis of our still somewhat fragmentary knowledge of their reproduction), the case of *Stephanopogon mesnili* shows that ciliates as well as foraminiferans include both homokaryotic and heterokaryotic species.

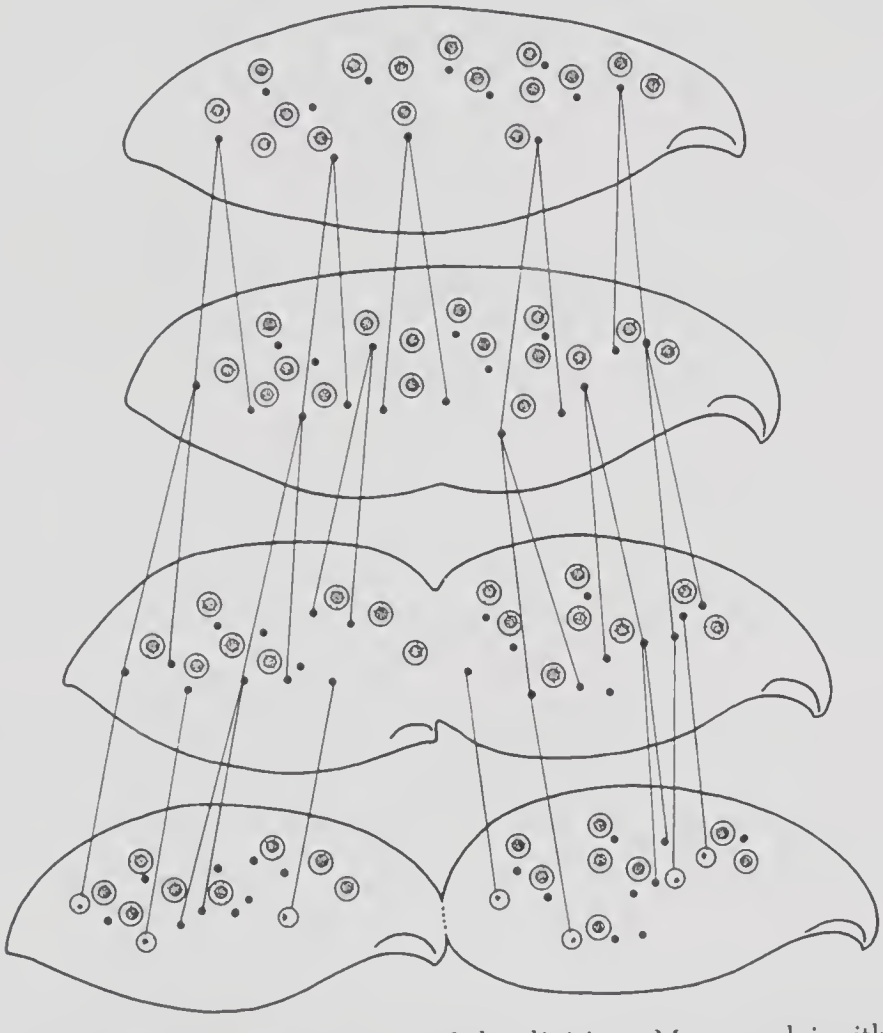


FIG. 29. *Loxodes magnus*. Diagram of the division. Macronuclei with nucleolus; micronuclei drawn as small black dots. After Raikov (1957).

Up to now, *Stephanopogon mesnili* is the only homokaryotic ciliate known.

As far as we know, all other ciliates have two different types of nuclei which, after O. Bütschli (1876) had first recognized their true nature, have been designated as macronucleus and micronucleus, respectively.

It became evident through the work of Fauré-Fremiet (1954) and of Raikov (1958, 1959a) that a distinction should be made between two kinds of nuclear dimorphism, which may be called primary and

secondary type. This distinction is based on the degree of polyploidy and the ability of the macronucleus to divide.

a. Primary type: diploid macronuclei incapable of division. Macronuclei of this type resemble the somatic nuclei of heterokaryotic foraminiferans in many respects: thus, their structure is similar to interphase nuclei in general; they contain nucleoli (usually one central nucleolus) and are unable to divide. Cell division is associated with a multiplication of micronuclei. Before the daughter cells separate, some of the micronuclei develop into a new set of macronuclei, this differenti-

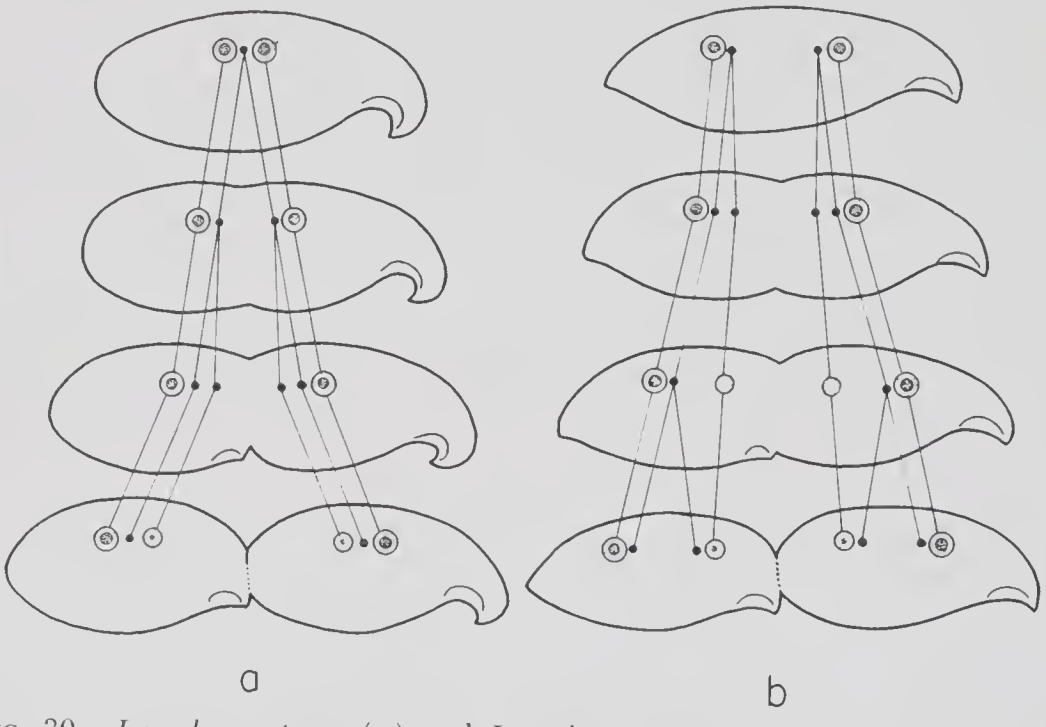


FIG. 30. *Loxodes rostrum* (a) and *Loxodes striatus* (b). Cell division. Macronuclei and micronuclei indicated as in Fig. 29. After Raikov (1957).

ation not being associated with any increase of chromosome number; the macronuclei are also diploid.

Figures 29 and 30 show the course of events in three species of the genus *Loxodes*. *Loxodes magnus* has many micronuclei and macronuclei, *Loxodes rostrum* possesses two macronuclei and one micronucleus while two nuclei of either type are characteristic of the third species, *Loxodes striatus*. The situation is similar in species of *Remanella*, *Geleia*, and *Trachelocerca* (genera from the rift system of marine sands: mesopsammon).

Figure 31 illustrates the very peculiar nuclear behavior found in *Trachelocerca phoenicopterus*. This ciliate has a composite nucleus consisting of six micronuclei surrounded by a "macronucleus" formed from six macronuclear anlagen (*a*). Division of the nuclear apparatus

begins with micronuclear mitoses leading to twelve micronuclei (*b*). The composite nucleus ruptures, each of the halves receiving six micronuclei (*c*,*d*). These divide once again (*e*). Six of the twelve daughter nuclei (*f*) represent the new set of micronuclei while the other six develop into macronuclear anlagen (*g*). After the remains of the old macronucleus have dispersed into the cytoplasm, the macronuclear anlagen

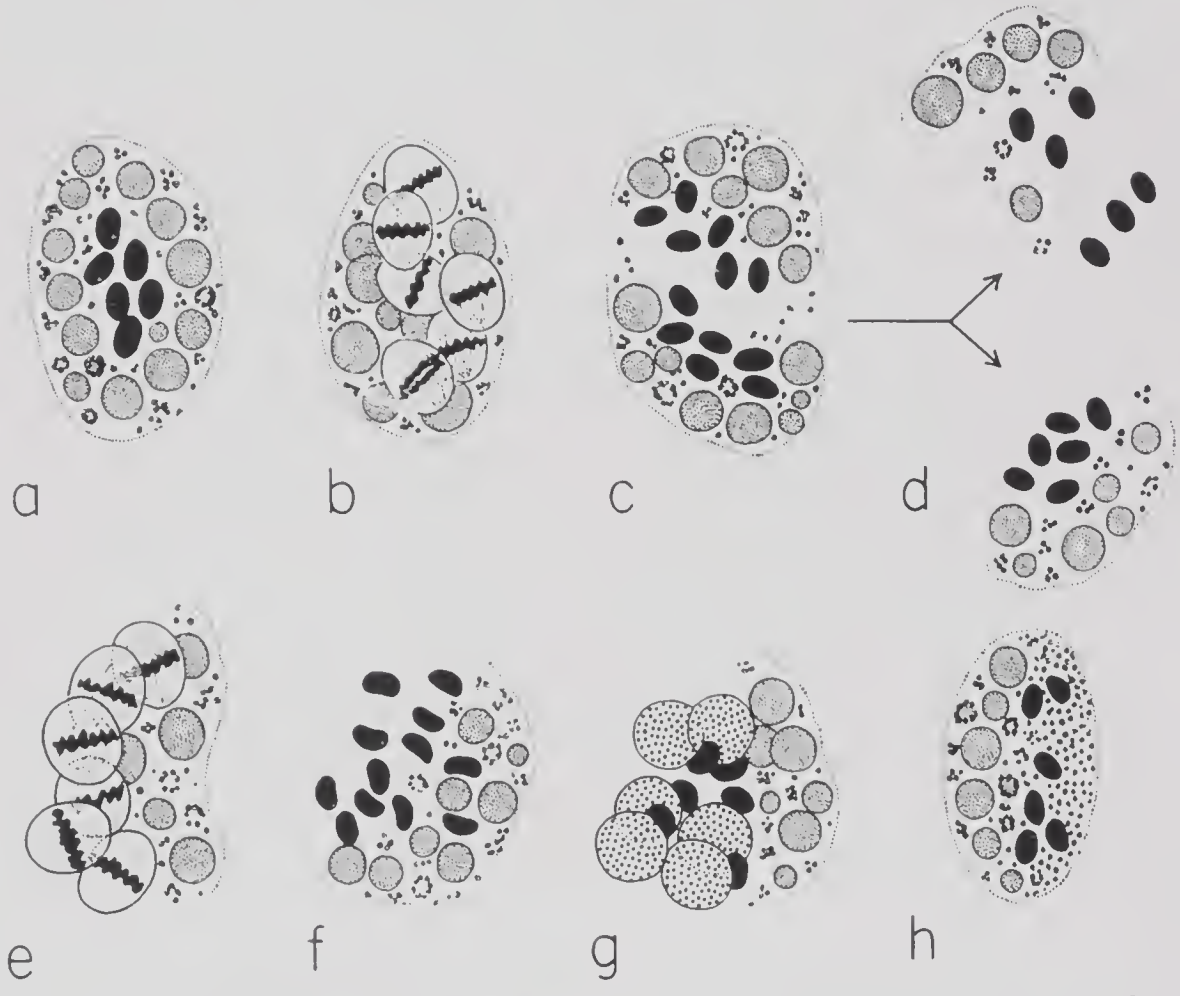


FIG. 31. *Trachelocerca phoenicopterus.* Behavior of the composite nucleus during division (see text). After Raikov (1958).

fuse and form a new composite nucleus which also includes the six micronuclei (*h*).

Investigation of the primary type of nuclear dimorphism is still in its beginning. With the exception of *Trachelocerca phoenicopterus* (Raikov, 1958), we know only the behavior of nuclei during asexual reproduction, and we may reasonably expect interesting insights from studies on sexual reproduction in these forms.

b. Secondary type: polyploid nuclei capable of division. Although Raikov's suggestion that the secondary type may have evolved from the

primary type seems plausible on general grounds, it is difficult to envisage just how this could have occurred. There is no problem in deriving a polyploid nucleus from a diploid one; however, daring additional

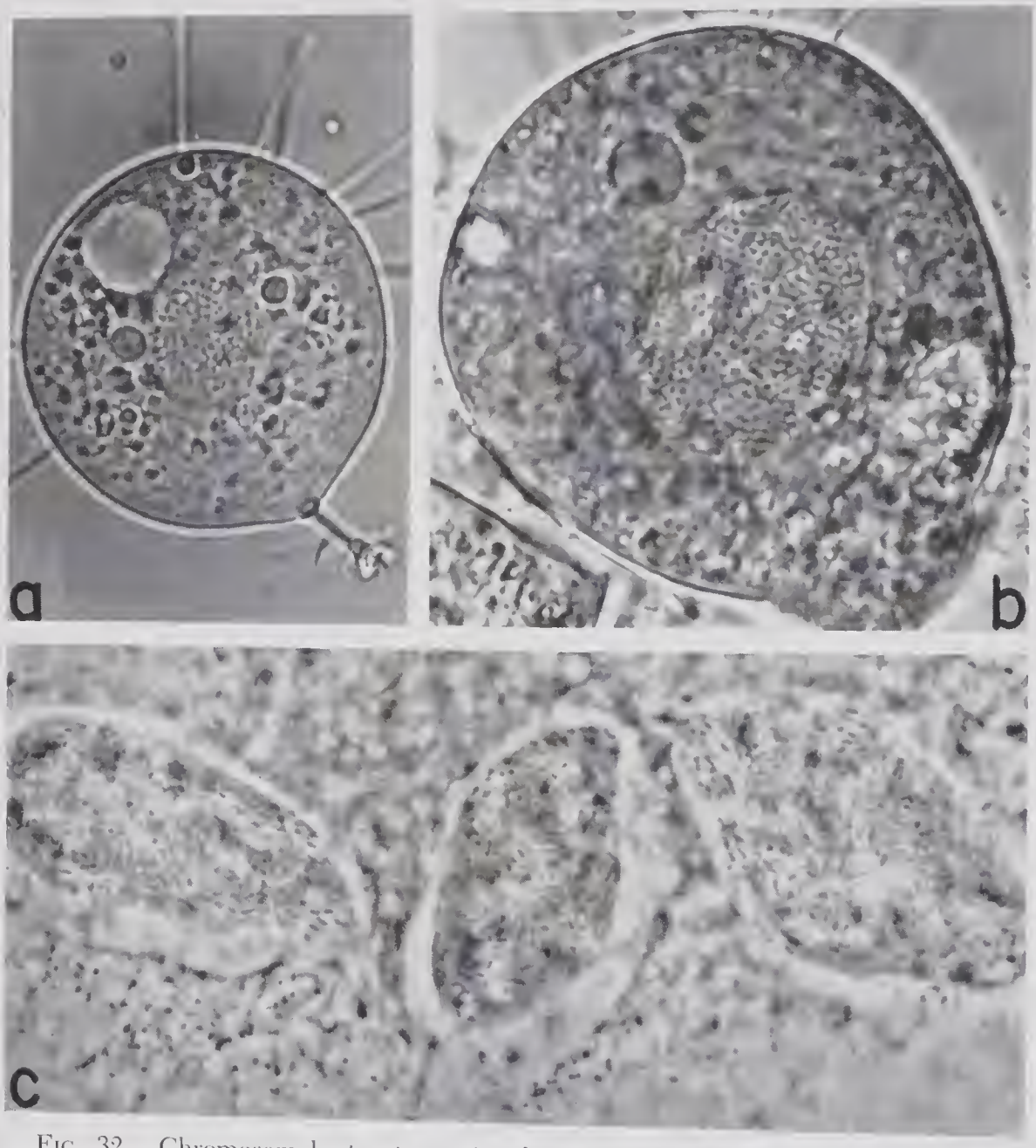


FIG. 32. Chromosomal structures in the vegetative macronucleus of ciliates. a, b. Living young and old specimen of *Tokophrya* sp. (suctorian). c. Three nodes of the macronucleus of *Loxophyllum meleagris* (euciliate), alcohol-acetic acid, unstained, phase contrast. a and b after Grell (1953e), c after Ruthmann (1963).

assumptions would seem necessary to explain how the polyploid nucleus could regain the ability to divide. Although the primary type is found only in some genera of holotrichs, the secondary type is characteristic for the majority of ciliates.

The consequences of a free permeability to *both* anions and cations in the presence of high concentrations of protein inside the cell was pointed out by Jacobs, restated by Davson, and was used as an argument that cation exchanges could not occur between cells and hypertonic or isotonic or hypotonic media, the reason being that if they did, the cells would swell indefinitely until they cytolyzed because of the colloid-osmotic pressure of the protein (dissociated or not) inside them. By 1940, we find permeability to cations no longer appearing as something to be denied, but as a step in a proposed mechanism of hemolysis or cytolysis. Suppose that a cell is normally impermeable to cations. No osmotic effects are produced by the unequal distribution of the anions in the presence of ionized protein, but if a lytic or injurious agent were to render the cells permeable to cations also, swelling would begin and continue. This hypothesis (the "dual hypothesis") was used to account for hemolysis by certain lysins (Davson and Danielli, 1938), for some of the behavior of ghosts (Davson and Ponder, 1938), and for photodynamic hemolysis (Davson and Ponder, 1940). In 1941, it was described by Wilbrandt under the name of colloid-osmotic hemolysis and was considered in much greater detail in a later and important paper by Wilbrandt (1948). The reasoning is still essentially the same: injury leads to a permeability to cations that is not normally present, and permeability to cations leads to a swelling that stops only when the cell cytolyzes.

What is unsatisfactory about the "dual hypothesis" is that it assumes a normal impermeability to cations that does not exist, and that it is indefinite in a quantitative sense. What occurs is better stated without using the terms permeable and impermeable. Granted that the protein inside the cell exerts a colloid-osmotic pressure, when the cell is in its normal environment this is presumably balanced by the concentrations and activities of the solutes in the environment being equal to the concentrations and activities of the solutes in the cell, including the protein. Under such circumstances, there can be no unidirectional movement of water and no change in the cell volume. There can be ion exchange without a volume change, however, provided that the loss of sodium ions, carrying some water with them, is balanced from the standpoint of concentration and activity by the gain of potassium ions carrying the same amount of water with them. This is usually what occurs, and the net effect of such an exchange has been called a "physiological impermeability to cations." When the transport mechanisms of the cell are impaired or when the structure of the cell is modified by the action of injurious substances, the restriction that potassium and sodium must be exchanged millimole for millimole may be removed, and the two ions

may move in different directions at different rates. If the ion inside the cell moves out more quickly than the ion outside moves in, the cell shrinks. If the reverse, it swells. To simplify the picture, suppose that an exchange of only one cation for another cation is involved and that the internal ion is called *I* and the external ion *E*. Let the rate of loss of *I* be large while the rate of entry of *E* is small. The cell will then shrink and the concentration of *I* becomes equal inside and outside. *E* will nevertheless enter the shrunken cell, however slowly, because the cell still contains protein with its colloid-osmotic pressure. The entry of *E*, which takes water with it, produces swelling that continues until the critical volume of the cell is reached and the cell cytolyzes. The obvious conditions that could prevent this happening would be a change in the state of the protein, a restoration of the restriction that the exchange must be millimole for millimole, or that there shall be no exchange at all.

The unsatisfactory feature of the hypothesis becomes clear when stated in this way. What happens to the cell volume depends essentially on the concentrations of *I* and *E* and on their rates of penetration across the surface of the cell. Although an approximate expression can be developed (Ponder, 1951c), what will happen under a special set of conditions involves so many constants, few of which can be determined independently, that it is possible to account for almost any volume change by making suitable assumptions. As might be expected, ion exchanges and red cell volume changes are poorly related in experiment, and there are some ion exchanges in which the result is a shrinking of the cell to its original volume after a stage of swelling and in which there is no evidence of the effect of the colloid-osmotic pressure of the contained protein. Further, the colloid-osmotic hypothesis indirectly carries with it an implication that the average cell hemolyzes at a particular critical volume. In the case of the red cell and the lysins digitonin and saponin, no such volumes are ever attained, and the possibility that lysins produce the condition for ion exchange, for colloid-osmotic hemolysis, and also affect the critical volume complicates the situation greatly.

The utility of the colloid-osmotic hypothesis, moreover, depends on the form of the question which it is expected to answer. Can the cation exchange observed be the cause of the hemolysis or cytolysis that occurs? The answer will generally be yes. We observe a cation exchange, sodium for potassium; will it necessarily result in hemolysis or cytolysis? The answer will often be no. Since we are not given values for all the constants, does the hypothesis allow us to make quantitative predictions? Generally, it does not. The "dual hypothesis" or the colloid-osmotic hypothesis is accordingly one which provides insight into many situations, but which frequently is not sufficiently specific.

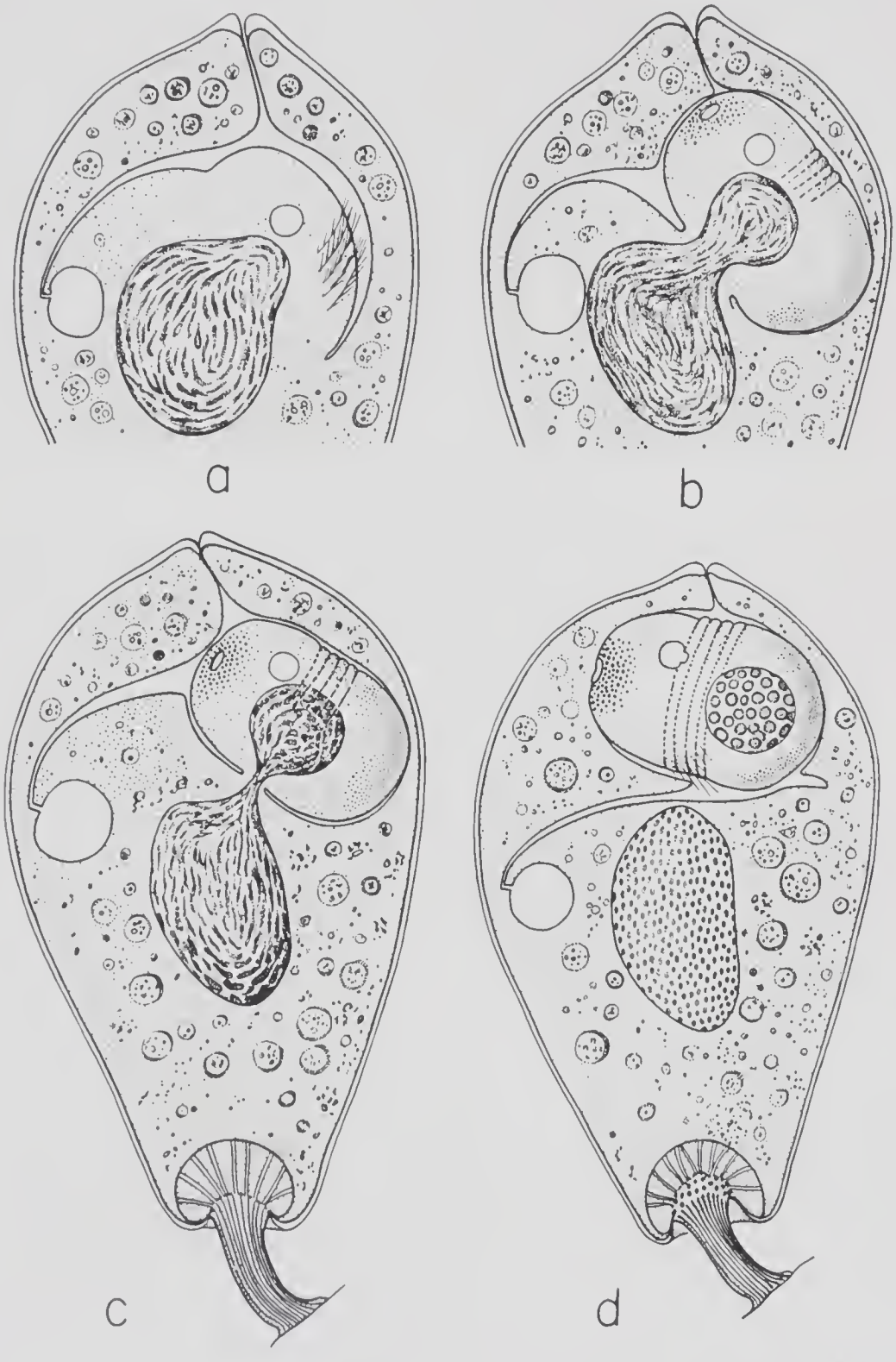


FIG. 34. *Tokophrya cyclopum* (suctorian). Endogenous budding, leading to formation of a swarmer within a brood chamber. After Collin (1912).

hypotrichous ciliates, there are two zones of structural change which are first evident at both ends of the macronucleus and progress toward its middle, where they finally meet. Gall could show both by spectrophotometry and autoradiography that DNA synthesis occurs within these reorganization bands. Regions already passed by the bands contained

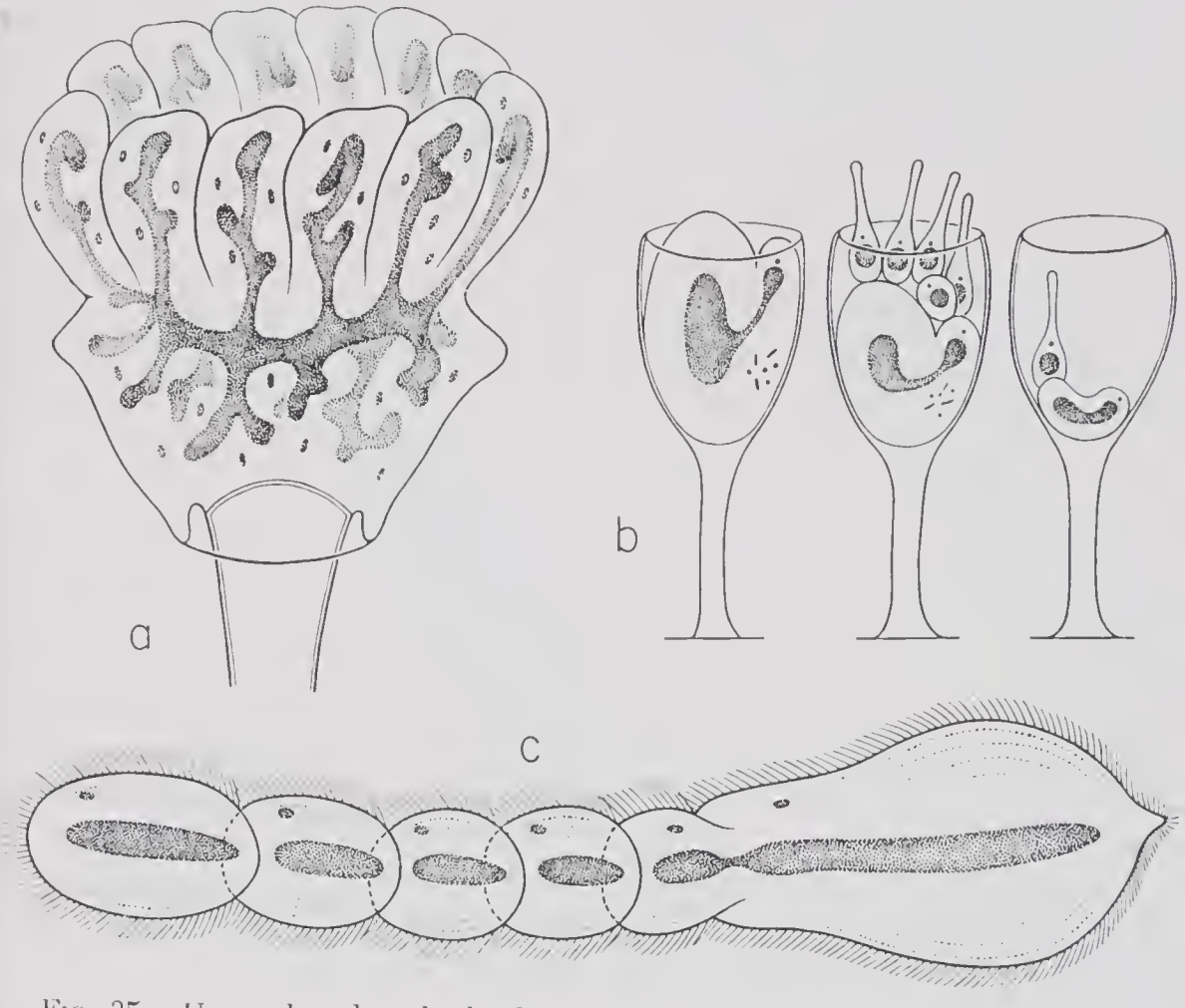


FIG. 35. Unequal and multiple division of the macronucleus. a. Simultaneous multiple division in *Ephelota gemmipara*. b. Successive multiple division in *Dactylophrya* stages of the parasitic suctorian, *Tachyblaston ephelotensis*. c. Unequal division in *Anoplophrya* (enciliate). After Grell (1953a).

twice the amount of DNA previously present (Fig. 36). The same relationship between reorganization bands and reduplication also holds for histones, the protein component known to be associated with DNA in the chromosomes of most organisms.

The mere fact that unequal and multiple divisions occur suffices to prove that the division of the macronucleus is not equivalent to mitosis, e.g., as found in polyploid nuclei of tissue cells. In any type of mitosis, the chromatids of all chromosomes would have to be distributed simultaneously to both daughter nuclei. On the other hand, the process

of macronuclear division would not seem to be adequately characterized by the term "amitosis." In the first place, amitosis is usually applied to cases where nuclei are simply pinched into two parts as described from differentiated tissues, e.g., the epithelium of the urinary bladder. The division of the macronucleus—be it equal, unequal, or multiple—is, however, an ordered process which must involve the distribution of whole genomes rather than single chromosomes. For this reason, the present author (Grell, 1950) proposed the term "genome segregation" in connec-

FIG. 36. *Euplotes curystomus.* Autoradiographs of the macronucleus after incorporation of H^3-thymidine. Incubation was 3 hours in (a) and 8 hours in (b). The arrow indicates the direction of movement of the reorganization band. After photomicrographs of Gall (1959)

tion with macronuclear division. It should be understood as a purely descriptive—not genetical—term.

The problem whether the distributional units of the macronucleus are diploid or haploid is still unsolved. Studies of Sonneborn *et al.* (1956), which were especially directed to solve this problem, indicated that heterozygous macronuclei of *Paramecium aurelia* retain their heterozygosity. Even after extensive aging, no loss of alleles occurred. Thus, the distributional units of the macronucleus seem to be diploid. This is consistent with Sonneborn's earlier assumption that a macronucleus contains diploid subnuclei which are capable of mitotic multiplication and are separated from each other by membranes of their own. On the other hand, there are no cytological data supporting this hypothesis. When-

ever chromosomes could be identified, they were found within a common nuclear space rather than grouped in subnuclei. Grell (1950) considered the possibility that the chromosomes of a genome might be attached to each other in linear order to form a "sammelchromosom" (compound chromosome) such as described by Cleveland (1949b, 1951a, 1953a) for some polymastigotes, e.g., *Leptospironympha*. Recent observations by Ruthmann (1962) in the ciliate *Loxophyllum meleagris* give some evidence for this (see Fig. 32c). Diploidy of the distributional units could be due to some sort of pairing of homologs. Whatever the answer may be, it is certain that the macronucleus consists of subunits which are separated from each other in the course of equal, unequal, or multiple divisions.

One might further consider that the subunits within a macronucleus can become differentiated from each other under certain conditions leading to the manifestation of different traits. This type of heterogeneity of the macronucleus might arise through activation of different pseudoalleles of a locus within the subunits. Thus, the subunits of a macronucleus may become determined for different mating types. During ordinary vegetative reproduction, these subunits may segregate, leading to the manifestation of different mating types in individuals from the same clone. In this way the phenomenon of selfing has been explained (Nanney and Caughey, 1955; Allen and Nanney, 1958).

If the subunits are heterozygous, one or the other of the two alleles may be differentially activated (Nanney and Dubert, 1960).

Despite the spectacular success of ciliate genetics, there is but little known concerning the functional meaning of nuclear dimorphism. The structure of the macronucleus, and especially its content of nucleolar substance which may either be present in form of free nucleoli or bound to chromosomes (Ruthmann and Heckmann, 1961; Ruthmann, 1963), suggests a prominent role in cellular metabolism. Under no circumstances can the cell survive the removal of the macronucleus. The micronucleus, on the other hand, is not essential for cellular metabolism. This is amply proved by the fact that in most ciliates amicronucleate strains show unlimited capacity to divide. Experiments of Sonneborn (1954) with *Paramecium aurelia* indicate that the genes of the micronucleus are normally inactive and that the phenotype of the cell is determined by the macronucleus alone. If these results can be generalized, the biological role of the micronucleus would be simply to permit genetic recombination during sexual processes, viz., conjugation and autogamy. However, this matter is not yet finally settled.

Not much is known about the conditions which lead to the differenti-

ation of such widely divergent types of nuclei. On the basis of cytological observations and centrifuging experiments, Nanney (1953) and Sonneborn (1954), working with *Tetrahymena pyriformis* and *Paramecium aurelia*, respectively, assume the determination of both types of nuclei to depend upon regional differences within the cytoplasm (see Nanney and Rudzinska, Volume IV of this treatise). However, it may be doubted that this idea can be generalized. Thus, a rather complex pattern of regional cytoplasmic differences would have to be present to account for the determination of new macronuclei after each cell division of *Loxodes magnus* (Fig. 29). In such cases it seems less forced to invoke feedback processes as discussed with regard to heterokaryotic foraminiferans.

One part of the hypothesis developed on page 44 was the idea that no additional somatic nuclei can arise because those already determined exert an inhibitory influence. This is also borne out by certain observations on ciliates. In *Epistylis articulata*, Seshachar and Dass (1953) observed that frequently the macronucleus may be lost through a disturbance of cellular division. However, this is not followed by death of the cell. After some time, a division of the micronucleus occurs; one of its products continues as a micronucleus while the other develops into a new macronucleus.[7] While in multimacronucleated ciliates numerous macronuclei are required to suppress the formation of additional macronuclei, the same feat is accomplished in unimacronucleated ciliates by a single differentiated nucleus. This difference may be due to the genetically determined variability of a threshold mechanism. In the first example the inhibitory influences of many nuclei have to add up while in the latter case a single nucleus exerts an inhibitory influence of sufficient strength. It is evident that such interactions between nuclei can occur only through the cytoplasm.

V. Nucleocytoplasmic Relationships

The relationships between nucleus and cytoplasm in Protozoa have been treated in the contributions by Brachet (Volume II) and by Nanney and Rudzinska (Volume IV) in this treatise. Nucleocytoplasmic interactions have also been variously discussed in the preceding pages. In the following we shall therefore consider only those topics that were not touched upon previously by ourselves or others. In doing so we cannot but order the facts in some—albeit arbitrary—manner.

[7] Observations of Sonneborn (1947) on macronuclear regeneration also confirm the idea that a differentiated nucleus exerts some inhibitory influences. Thus, fragments of the old macronucleus formed at conjugation will develop into new macronuclei only if the formation of a new macronuclear anlage is suppressed.

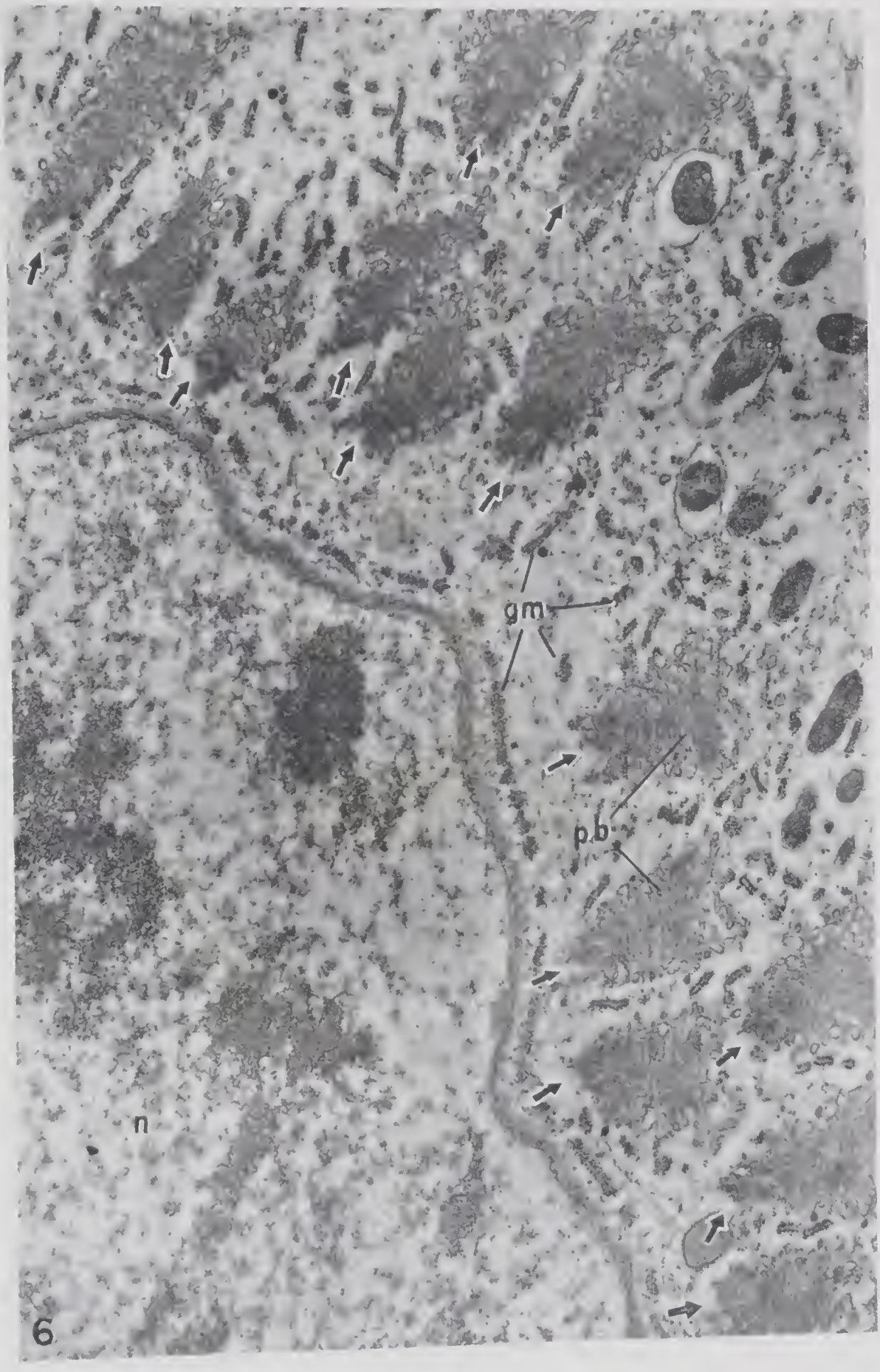
A. The Influence of the Nucleus on the Cytoplasm

1. Cytological Evidence

Aside from instances where nuclear contents are discharged into the cytoplasm either through total disintegration (e.g., somatic nuclei of heterokaryotic foraminiferans and ciliates, certain postmeiotic and postzygotic nuclei in both groups) or through partial disintegration (nuclei of gamonts of monothalamic foraminiferans and of gregarines), there are only few cytological observations of nuclear structures passing into the cytoplasm. During macronuclear divisions of some ciliates, chromosomal material ("extrusion chromatin") is regularly shed into the cytoplasm, e.g., in *Conchophthirius mytili* (Kidder, 1933). Similar phenomena have been observed during divisions within the cysts of Colpodidae (Burt *et al.*, 1941; Padnos, 1962). In some respects, this reminds one of the elimination of chromatin in certain metazoa such as copepods. However, it is well to recall that the macronucleus is polyploid. The extruded material may thus correspond to complete genomes whereas in the metazoa just mentioned DNA-containing material is extruded from diploid nuclei.

Passage of *nucleolar substance* into the cytoplasm has been described repeatedly. It is a regular feature in the macrogamonts of coccidians and was interpreted as a "maturation phenomenon" by earlier authors. It occurs probably just once at the end of the growth phase when sporogony, a phase of nuclear multiplication not associated with increase of cellular mass, commences. It has, however, been maintained that formation and extrusion of nucleoli can occur repeatedly (see Scholtysek, 1954). In the macronuclei of the ciliate *Geleia nigriceps*, extrusion of the nucleolus seems to occur regularly (Raikov, 1959b). In this case it appears to be an expression of the metabolic activity of the nucleus and is one means of passing nucleolar material into the cytoplasm, although it is surely not the only way by which this is accomplished.

As is well known, major portions of cytoplasmic RNA are bound to ribosomes of the ergastoplasm. There are reasons to believe that the latter are either formed by the nucleus itself or, at least, with its aid. An electron microscope study of Grimstone (1959) with *Trichonympha* is of special interest in this connection. As shown in Fig. 37, there is a chain of membranes next to the nuclear membrane and parallel to it. Both types of membranes are studded with small particles in the size range of ribosomes, those on the outer nuclear membrane occurring mainly at the rims of the nuclear pores. Grimstone believes that the membranes are formed in the vicinity of the nuclear envelope while their granular


gm
pb
n
6

covering may derive from the nucleus itself. After formation, the granular membranes (*gm*) are given off into the cytoplasm and become abundant everywhere in the cytoplasmic area. It seems to be one of their functions to participate in the formation of the parabasal bodies, which are shown in cross sections in Fig. 37 (*Pb*). The latter consist of double lamellae forming a pile of flattened sacs and the parabasal filament, a protein fiber which is always found at the lamellar surface directed toward the nucleus. As pointed out by Grassé (1956), this structure is reminiscent of the Golgi apparatus. The outer double lamellae are evidently continuously pushed off as vesicles into the cytoplasm while new lamellae are formed inside the parabasal body. According to this interpretation, which is also supported by starvation experiments, the parabasal bodies are to be considered a steady-state system which is maintained through continuous addition of granular membranes formed under the influence of the nucleus. The particular role which this system may, according to Grimstone, fulfill in cellular physiology cannot be considered at this point.

However suggestive, the electron microscope researches cited do not constitute final proof of nuclear influences upon the formation of cytoplasmic structures. More direct evidence for the passage of nuclear material into the cytoplasm stems from observations of vesicles and blebs which are pinched off from the nuclear surface. The work of Brandt and Pappas (1959) with the ameba *Pelomyxa carolinensis* indicates that mitochondria—whose origin is still doubtful (see Novikoff in Volume II of this treatise)—may originate in connection with some such process. Their electron micrographs show distinct tubules within vesicles which are being pinched off from the nuclear envelope. It should be pointed out, however, that such appearances are restricted to resting nuclei right after reconstruction. Interrelationships of DNA and mitochondria were also discussed in this treatise by Mirsky and Osawa (Volume II). These authors draw also attention to electron microscope findings suggesting the kinetoplast of trypanosomes as a site of origin for mitochondria.

Nuclei were formerly uncritically credited with the formation of certain cell organelles. Thus, Tönniges (1914) gave a detailed description of the elaboration of trichocysts by the macronucleus. Such phantasies

FIG. 37. *Trichonympha* (polymastigote flagellate). Electron micrograph of an ultrathin section showing the relationship of the nucleus (*n*), granular membranes (*gm*), and parabasal bodies (*pb*). The filaments of the parabasal bodies are indicated by arrows: note that they are all directed toward the nucleus. Magnification: × 23,000. After Grimstone (1959).

which, on occasions, appear even today are not apt to encourage confidence in protozoological cytology.

It is certain, however, that the centrioles of polymastigotes can display morphogenetic activity even if located within the nucleus (see page 15). The cell organelles formed by them (flagella, axostyles) penetrate the nuclear envelope but remain permanently connected with the nucleus because of the intranuclear location of the centrioles.

2. *Cytochemical Evidence*

Definite statements concerning the influence of the nucleus upon the cytoplasm can be made on the basis of cytochemical methods which involve labeling of substances passing from the nucleus into the cytoplasm. Another obvious approach is to remove the nucleus and test the synthetic capacities of the cytoplasm alone. These experiments which were mostly conducted with *Amoeba proteus* have already been described in detail by Brachet (Volume II in this treatise). In this context, we should only like to draw attention to autoradiographic studies of Prescott (1959, 1960) indicating that, in amebae, RNA synthesis seems confined to the cell nucleus.[8]

3. *Genetical Evidence*

There are two different methods to prove that a given cellular trait is dependent on the nucleus. The first is by nuclear transfer between different races or species. Working with amebae, this technique has been applied by Lorch and Danielli (1950) and Danielli (1955, 1959a, b). Since their results were described by Brachet (Volume II in this treatise), we shall only call attention to them in this context.

The second is by crosses between races of a species. This is feasible only in sexually reproducing organisms. In Protozoa, genetical investigations of this kind have so far been possible only in phytomonads and in ciliates.

The *phytomonads* are haploids with zygotic meiosis. Since in chlamydomonads all gones emerge as a rule from the zygote cyst, they are particularly well suited for tetrad analysis. The inheritance of many morphological, physiological and biochemical traits has been followed in species of the genera *Dunaliella* (Lerche, 1937) and *Chlamydomonas*

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[8] Presumably, this conclusion applies to both types of RNA of known function, i.e., high molecular weight messenger RNA engaged in transmission of genetic information and low molecular weight transfer RNA transporting amino acids to ribosomes. However, it may be doubted that all cellular RNA is, in every case, of exclusively nuclear origin.

(see Levine and Ebersold, 1960, for review). Aside from a few exceptions which necessitated the postulate of extrachromosomal factors (Sager, 1960), tetrad analysis led to results expected on the basis of Mendelian inheritance. Aside from this result of general genetic interest, these investigations have not led to any new insights into the relations between nucleus and cytoplasm.

The situation is different in *ciliates*, which, in contrast to phytomonads, are diploids with gametic meiosis. Because of their peculiar mode of sexual reproduction they are well suited for investigation of nucleocytoplasmic interactions. Without going into details which may be looked up in reviews (Sonneborn, 1947; Beale, 1954), I should like to discuss some results which seem to me of particular interest in this connection.

The most important discovery of ciliate genetics bearing upon the problem of cell differentiation seems to me that *cells of the same genetic constitution can realize different heritable traits.* This was first proved for the mating types.[9] Within each variety of *Paramecium aurelia* the cells, though genetically alike, can realize two different mating types and, in variety 1 of *Tetrahymena pyriformis*, even seven different types. In both instances it could be shown that determination of the mating type occurs in a sensitive period during the development of the macronuclear anlagen. In this period, the type to be realized can be influenced by external conditions. In *Paramecium aurelia*, for instance, increase of temperature favors the appearance of the evennumbered mating types. Once the sensitive period is passed, the ability for realization of a given mating type is inherited without further change.[10]

In *Paramecium aurelia* the role of the cytoplasm in the determination of mating types is very different in the varieties belonging to groups A

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[9] This does not hold, of course, for cases where mating types are directly determined by the genetic constitution, as in *Euplotes patella* (Kimball, 1942), *Euplotes rannus* (Heckmann, 1961, 1963), and *Paramecium bursaria* (Siegel and Larison, 1960).

[10] It is certain that the spectrum of mating type potentialities is controlled by a *mt*-locus. Alleles changing this spectrum have been found in *Paramecium aurelia* (Sonneborn, 1939; Butzel, 1955) as well as *Tetrahymena pyriformis* (Nanney *et al.*, 1955). However, it is possible that the *mt*-locus is complex, consisting in *Paramecium aurelia* of two (I+, II+ of variety 1) and in *Tetrahymena pyriformis* (variety 1) of seven (I+, II+, III+, IV+, V+, VI+, VII+) functional subunits or pseudoalleles; only one of these is activated during the sensitive period of macronuclear development (e.g., in *Paramecium aurelia* I+ II+ or I+ II+). According to this interpretation (Grell, 1956), the determination of mating types would furnish a typical example for differential gene activation which would, in this case, be irreversible.

and B. In the latter, the cytoplasm is of much greater importance than in the former. In group B varieties, macronucleus and cytoplasm exert influences upon each other. The macronucleus causes a certain condition in the cytoplasm which is specific for the given mating type. The cytoplasmic state, in turn, conditions after conjugation or autogamy the newly developing macronuclear anlagen for the same mating type. It is

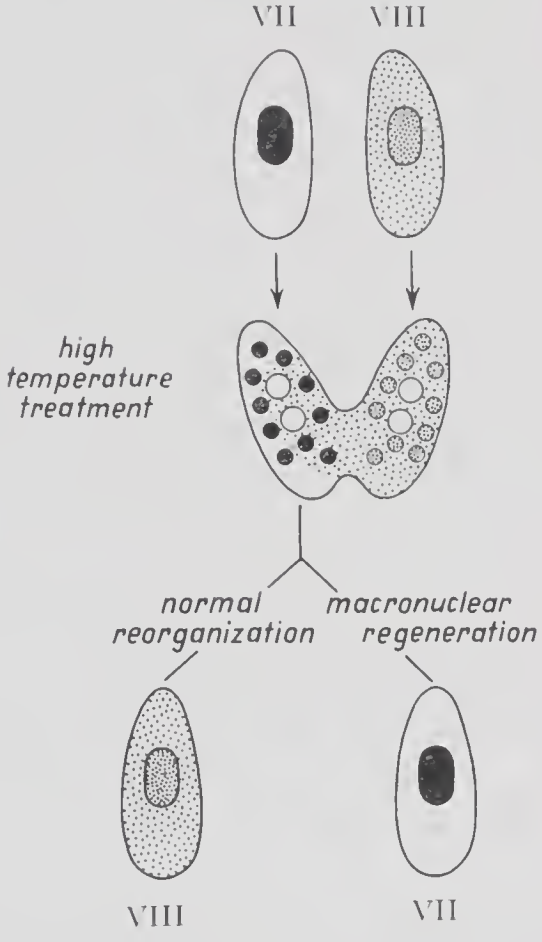

FIG. 38. Sonneborn's experiment on inheritance of mating types VII and VIII (variety 4, group B), after conjugation involving cytoplasmic exchange, normal reorganization, and macronuclear regeneration. Only macronuclei or macronuclear fragments (black and stippled) and anlagen (white) are shown. After Beale (1954), modified.

for this reason that in most exconjugants or exautogamonts of group B varieties the same mating type is realized as in those conjugants or autogamonts from which they originated.

These relationships between nucleus and cytoplasm were largely clarified through an experiment by Sonneborn (1954) which is diagrammed in Fig. 38. After conjugation of animals belonging to mating types VII and VIII (variety 4) pairs were isolated in which an extensive exchange of cytoplasm had taken place. The exconjugants were subjected

to temperatures high enough to delay the development of macronuclear anlagen without entirely suppressing them. From these, two kinds of clones could be derived: (1) clones from individuals in which a normal macronuclear reorganization had taken place, and (2) clones from individuals whose macronucleus derived from a regenerated fragment of the old macronucleus (see page 51). If the conjugant was of type VII then clones of the first sort changed to VIII. This could be due only to a determining influence of the type VIII-cytoplasm upon the developing macronuclear anlagen. In clones of the second kind the same mating type was realized as in the conjugant whose macronuclear fragment had regenerated. Although, after massive exchange of cytoplasm at conjugation, exconjugants contain cytoplasm of both types VII and VIII, the clones retained their previous mating type even after autogamy. This means, however, that the macronucleus, in accordance with its determination, can call forth a condition within the cytoplasm which is passed on and determines the new macronuclear anlage after autogamy.

Sonneborn's experiment shows not only that mutual influences of nucleus and cytoplasm are operative in determining the mating type in varieties of group B, but also that the ability of the macronucleus to realize a given mating type is not lost when it fragments at conjugation and subsequently regenerates.[11]

B. The Influence of the Cytoplasm on the Nucleus

1. Induction of Division

a. Binary division. R. Hertwig (1908) was among the first biologists who thought about the relations between nucleus and cytoplasm in quantitative terms. He assumed a definite ratio between nuclear and cytoplasmic mass to be characteristic for each protozoan cell, called "Kern-Plasma-Relation" ("karyoplasmic ratio" cf. Wilson) by him. This ratio would be "normal" only right after cell division. During cellular growth, the karyoplasmic ratio would be altered in favor of the cytoplasm. While the nuclear mass increases only slightly at first, the cytoplasm increases rapidly and continuously. This change of the karyoplasmic ratio during growth would cause a "tension" within the cell leading to the release of nuclear and cell division.

With some ciliates, it could be shown that the course of mass increase of nucleus and cytoplasm is indeed divergent as supposed by

[11] If this property depends on the activation of a pseudoallele within the *mt*-locus (see footnote 10), then it would mean that this state of gene activation would be retained in a "differentiated" macronucleus.

Hertwig (see Popoff, 1908, 1909). Yet, there are also examples of the opposite among ciliates. In suctorians, for instance, the course of nuclear and cytoplasmic growth is rather more convergent. The division of the macronucleus, though equal in most euciliates, is unequal in suctorians (Figs. 34 and 35). Both the mother cell and the mother macronucleus form one or several small buds of corresponding nucleocytoplasmic mass ratios. After metamorphosis, the young suctorian grows continuously, nuclear and cytoplasmic mass increasing parallel to each other. Presumably, the increase of macronuclear mass is due to endomitotic polyploidization (see page 51).

Aside from the fact that such cases are difficult to reconcile with the idea of "karyoplasmic tension," the macronucleus should not be considered a "typical" nucleus in the general sense because of its endopolyploidy. Its increase in volume is not comparable to prophasic swelling as seen in ordinary cell nuclei. Furthermore, its division is in many cases connected with specific changes in morphology unknown from other nuclei. One example of this is furnished by *Stentor coeruleus*, whose macronucleus is beaded like a string of pearls. Before division it loses its beaded structure and condenses into a uniform mass. With the onset of cell division, it stretches again and resumes its former appearance, the number of beads being increased. By nuclear transplantation, De Terra (1960) could show that these morphologieal changes are largely dependent upon cytoplasmic conditions. If a condensed nucleus is transplanted into a nondividing cell, it remains in the condensed state. When transplated into a cell which is about to divide and hence has a condensed nucleus of its own, both the transplanted and the original nucleus will subsequently stretch together. Other experiments, which cannot be deseribed in detail, showed that the division of the macronucleus itself, which consists simply in elongation of the thread connecting two median beads to the point of rupture, is dependent upon the existence and the position of the cleavage furrow.

The nature of the cytoplasmic conditions determining the morphological changes and the division of the macronucleus are unknown. We ought to mention, however, that cytoplasmic reorganization which occurs from time to time in *Stentor coeruleus* entailing renewal of the membranelle band and other differentiations of the peristome is also accompanied by morphological changes of the macronucleus exactly like those associated with cell division (Schwartz, 1935).

There is undoubtedly some truth in Hertwig's ideas, because nuclear and cytoplasmic division is frequently initiated when the cell has reached a certain *limit size* (critical mass, Mazia) corresponding to its genetically

determined biological norm. It was Hartmann (1926, 1928) who first showed through his experiments involving repeated amputation of portions of the cytoplasm of *Amoeba proteus* that it is a cytoplasmic condition established after the cell reaches its limit size which induces division, not the process of aging as such. In this way, "immortality" of the individual cell could be attained. In one of his experiments, a single ameba was kept alive through 130 amputations and regenerations for a span of 4 months without divisions while its clonal sisters passed about 65 divisions in the same period.

Usually nuclear and cell divisions in Protozoa are synchronized only if the cells are uninuclear. In multinuclear species, simultaneous division of all nuclei before cell division is exceptional and occurs, for instance, in the ameba *Pelomyxa carolinensis* (Kudo, 1947) and in opalinids of the genus *Zelleriella* (Chen, 1948).

As a rule, nuclear divisions are concomitant with cellular growth, or else a growth phase with a single nucleus is succeeded by a period of nuclear multiplication leading to multiple division of the cell.

b. Multiple division. Multiple divisions are found in many amebae, radiolarians, foraminiferans, and sporozoans. As an example, the coccidian *Eucoccidium dinophili* (Fig. 39) of the archiannelid *Dinophilus gyrociliatus* will be discussed. From other coccidia it is distinguished by its extracellular development in the body cavity of the host and the absence of schizogony. As soon as the sporozoites (*1*) have penetrated into the body cavity (*2*), the growth phase sets in. In the case of microgamonts, this lasts only for a short while. Nuclear multiplication starts early, leading to 12–32 nuclei which become the nuclei of microgametes (*3–6*). In macrogamonts, the growth phase is considerably more extended, and, after reaching a certain size, they assume an oval shape and are fertilized by microgametes (*9*); in this way the zygote is formed. After nuclear divisions set in, this is called "oocyst" in accordance with general usage. The first division of sporogony (*10, 11*) leads to chromosomal reduction (zygotic meiosis). Further nuclear divisions at the surface of the cell body follow (*12*), and finally the whole cytoplasm is subdivided to form the spores, which are at first uninuclear (*13*). After the oocyst membrane has ruptured, further divisions occur within each spore leading to a total of 6 sporozoites (*14*). These spores become free after death of the host and are ingested by other worms.

If the infection of the host is weak, only macrogamonts are formed. These can develop parthenogenetically; i.e., there is no fertilization and no meiosis. Instead of the usual alternation of generations, development consists then only of the growth phase of a single cell (sporozoite →

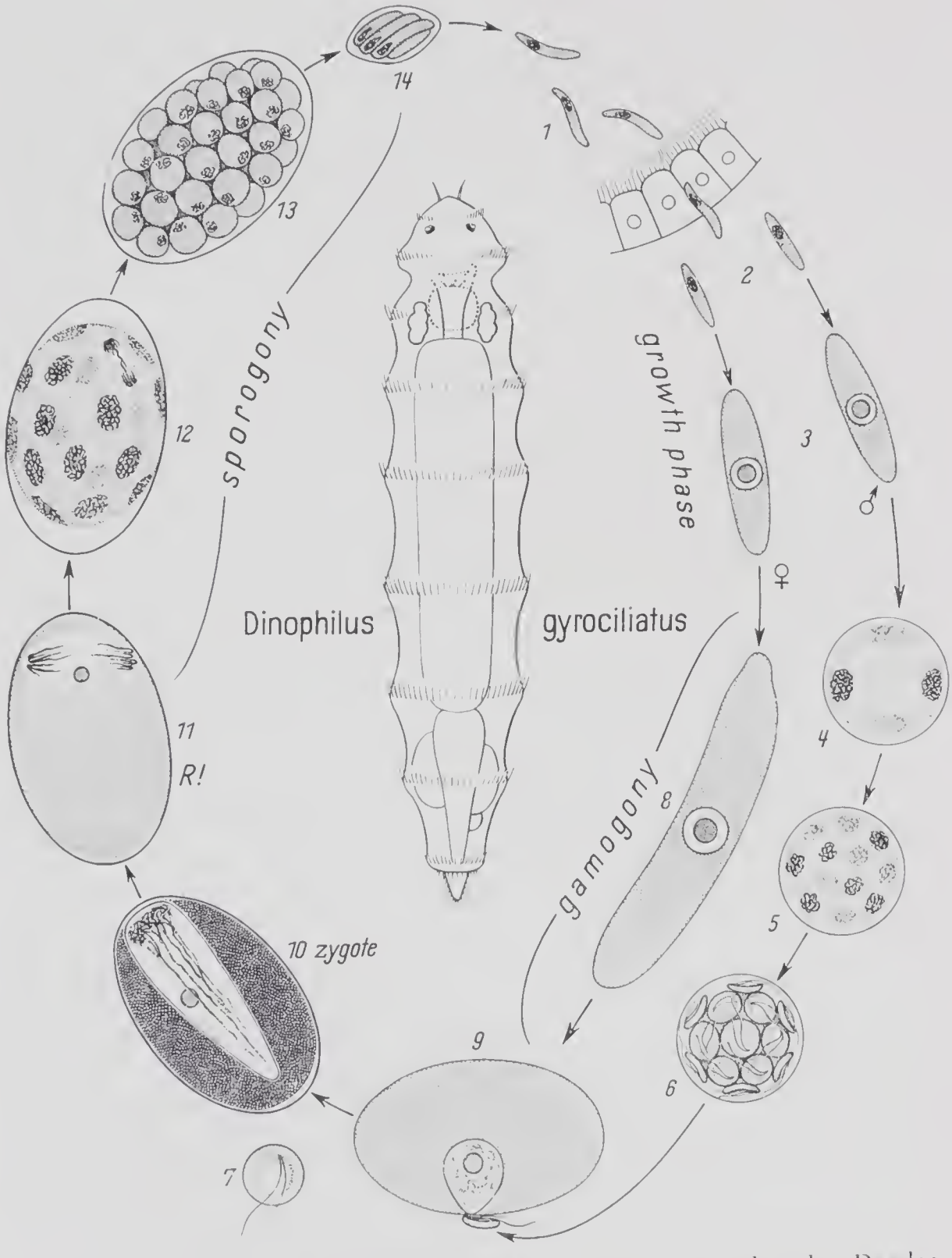

FIG. 39. *Eucoccidium dinophili* (sporozoan). Developmental cycle. Development is extracellular within the body cavity of the marine archiannelid *Dinophilus gyrociliatus*. After ingestion the spores rupture and the sporozoites penetrate the intestinal wall (*1*, *2*). In the body cavity the sporozoites grow and differentiate directly into gamonts (*3*). The macrogamonts are large and cigar shaped (*8*); the microgamonts are small and round. The microgamonts undergo multiple division

macrogamont) and its multiple division (sporogony). Hence neither meiosis nor fertilization are necessary for the release of multiple division. These cells cannot grow indefinitely, but, after reaching a certain limit size, they must enter the period of nuclear multiplication. This can be shown if hosts are infected with only a single spore. Even if only one sporozoite develops in the body cavity—which is frequently the case in one-spore infections—it will not grow beyond a certain length (about

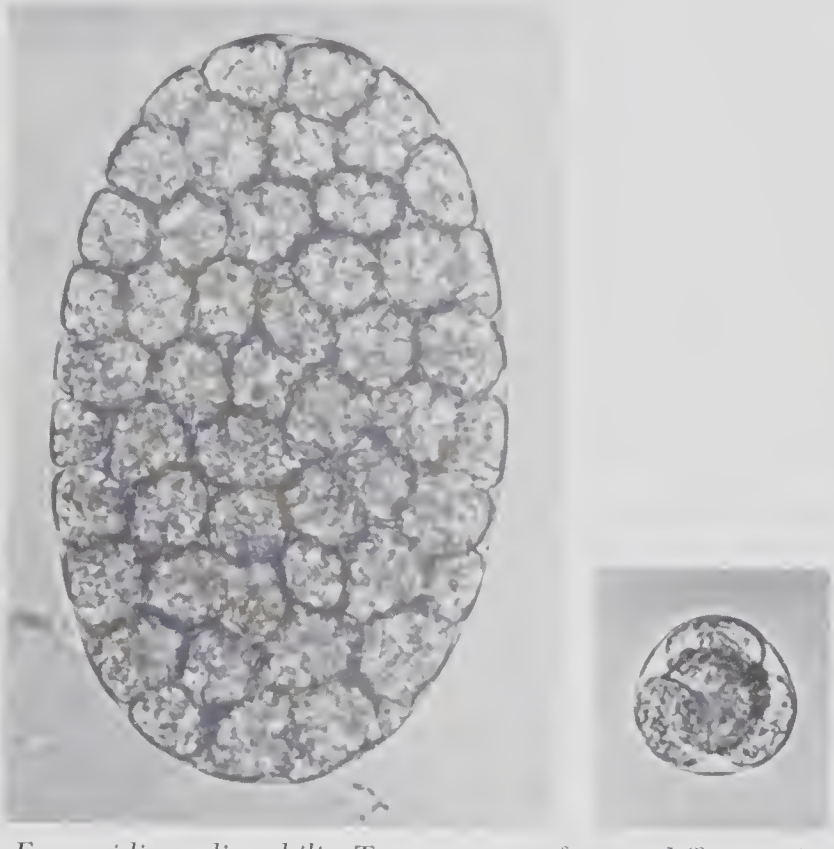

FIG. 40. *Eucoccidium dinophili.* Two oocysts of very different dimensions containing spores of the same size. After Grell (1953d).

150 μ) before entering sporogony. This size limit can, however, be modified by external factors: it is reached earlier in strong infections than in weak ones. In the first case, there will be small oocysts with occasionally as few as four or even two spores, while in the second case large oocysts with many (about 250) spores are formed. While the size

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and form about 12–32 microgametes (*4–6*), each bowl shaped with two flagella of different length (*7*). The macrogamonts become rounded macrogametes. After fertilization (*9*) the synkaryon of the zygote stretches to form a so-called "fertilization spindle" (*10*). Sporogony begins with meiosis (*11*) and leads to the formation of uninuclear spores (*12, 13*). The oocyst ruptures and the spores are liberated into the body cavity. Six sporozoites are formed within each spore (*14*). The spores are set free after death of the host. After Grell (1953d).

of the oocysts is thus variable, that of the spores is always about the same (Fig. 40). The constancy of spore size is regulated by the number of nuclear divisions preceding their formation. Experiments with colchicine (Grell, unpublished) showed a direct correlation between the reduplication of the chromosomes and the mass of the cytoplasm. If infected worms are transferred into a solution of colchicine (~0.2% in sea water), the formation of the divisional apparatus can be suppressed. Instead of a sequence of nuclear divisions, endomitotic reduplication of chromosomes takes place leading to a large polyploid nucleus instead of many haploid nuclei (Fig. 41). It becomes evident at once that small

FIG. 41. *Dinophilus gyrociliatus* (archiannelid) infested with *Eucoccidium dinophili*. From an experiment involving colchicine treatment during the growth phase of macrogamonts. The oocysts contain a single polyploid nucleus instead of many haploid nuclei at the end of sporogony. From a Feulgen-stained slide.

cells form a small nucleus while large cells will also contain a large polyploid nucleus. Spectrophotometry of the DNA content of these nuclei would probably show that the chromosomes divide as often in colchicinated material as in normal sporogony.

Due to colchicine treatment, oocysts with a polyploid nucleus are unable to form spores. Treatment of the spores used for infection with X-rays leads to rather surprising results at the ensuing sporogony divisions (Grell, 1955; Stössel, 1961). The sporozoites from these spores grow up normally, latent defects showing up only at sporogony. A breakage-fusion-bridge cycle may be set up and, after some divisions, may cause difficulties in the separation of daughter nuclei leading to "restitutional nuclei" of different sizes. This leads to irregular subdivision of the cytoplasm in oocysts with radiation defects (Figs. 42 and 43). However, certain rules seem to be obeyed: (1) cytoplasmic regions are

delimited only around isolated masses of chromosomal material; (2) the amount of cytoplasm is correlated with the amount of chromosomal material; (3) the portions of the ooeyst formed by subdivision do not normally develop into spores and ultimately degenerate (for exceptions, see Stössel, 1961). While multiple division as such depends only upon the

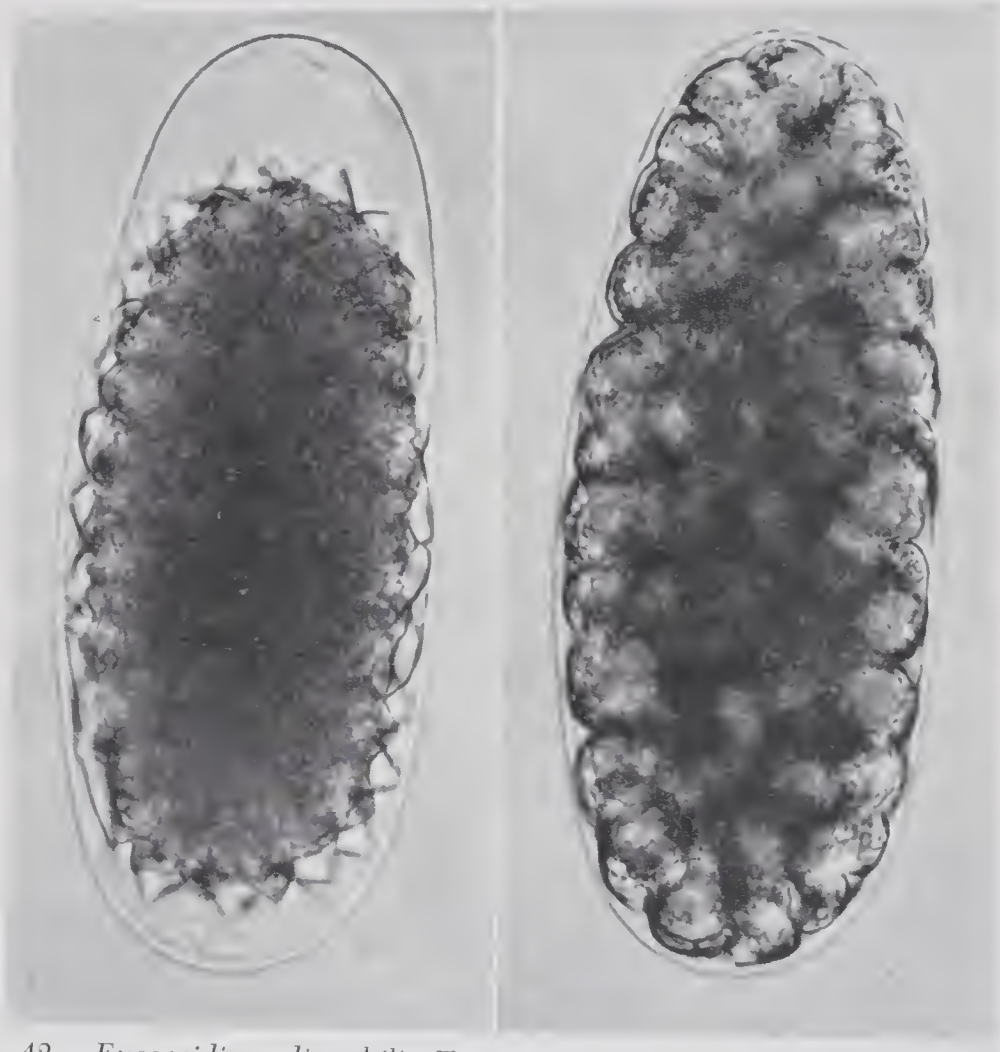

FIG. 42. *Eucoccidium dinophili.* Two successive photomierographs of the same living oocyst from a hanging drop preparation. The pointed projections in the left-hand picture indicate the superficial positions of centrioles during the sporogony divisions. Fully formed spores (at right) are of equal size and fill the whole cavity of the oocyst. After Grell (1953d).

presence of isolated masses of chromosomal material, not on the existence of balanced genomes, the seeond phase of sporogony (formation of spores and sporozoites) requires the existenee of normal haploid nuclei.

c. Meiosis. As a rule, meiosis as a developmental phase is an obligatory and integral part of the proeesses connected with sexual reproduction. Evidence for substances supposed to trigger meiosis is therefore questionable if other phases of sexual reproduction preeede it.

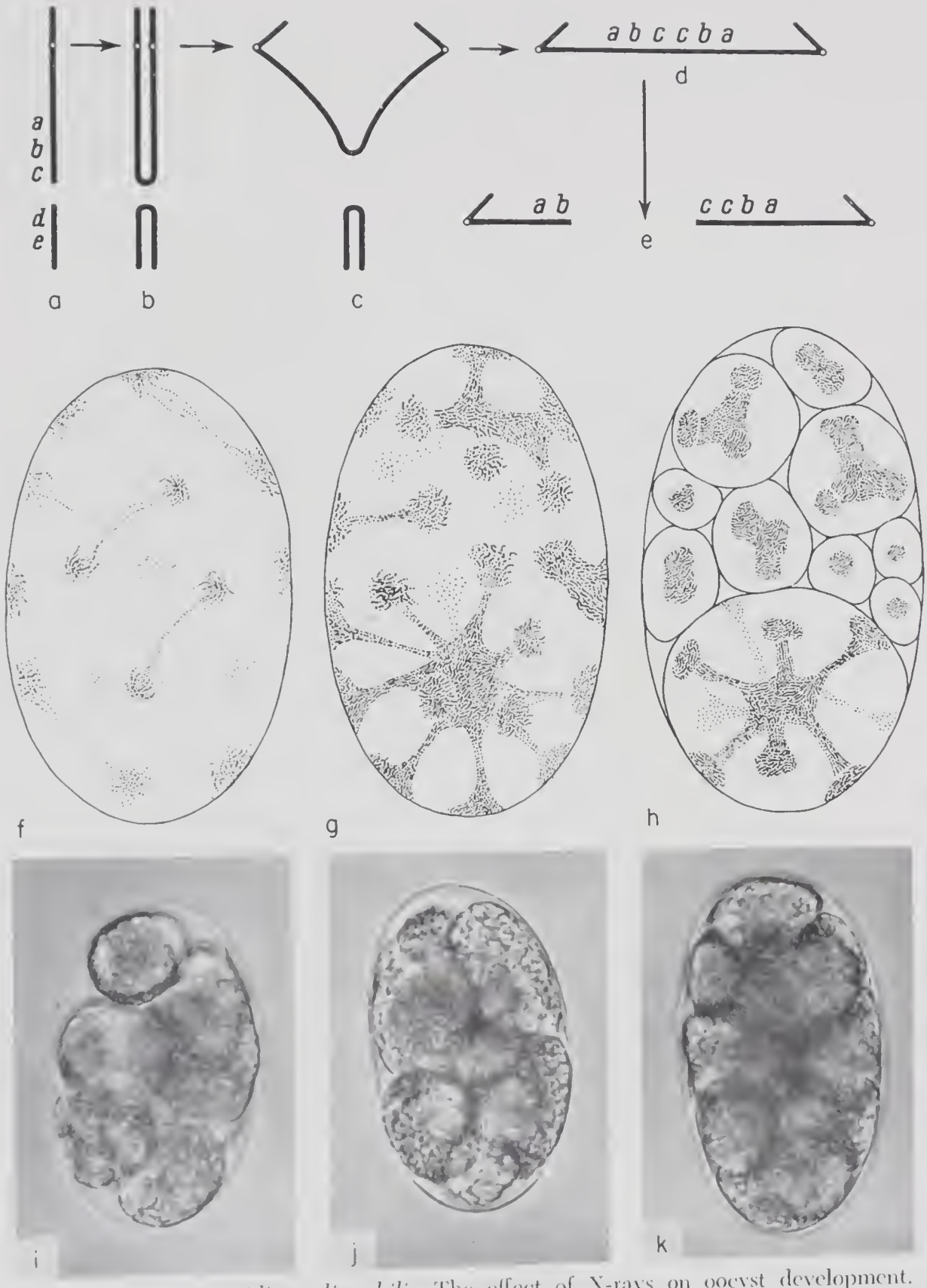

FIG. 43. *Eucoccidium dinophili*. The effect of X-rays on oocyst development. a–e. Breakage-fusion-bridge cycle. f, g. The resulting failure of the chromosome masses to separate during sporogony division. h. Portions of cytoplasm are delimited around isolated chromosomal masses only. i–k. Photomicrographs of oocysts containing plasma masses of different size (corresponding to h). After Grell (1955).

Ecdysone, for instance, if applied to diploid polymastigotes (Cleveland, 1959; Cleveland and co-workers, 1960), may be responsible for the conversion of "vegetative" cells to gamonts, but most likely not for the onset of meiosis as such. In other words, the action of ecdysone on diploid polymastigotes would not seem different from its effect on haploid species, viz., initiation of sexual reproduction.[12]

Meiosis is probably always preceded by a specific state of the cytoplasm. This can be demonstrated with foraminiferans which begin meiosis only after the agamonts have reached a certain critical size. In the homokaryotic species *Patellina corrugata*, the number of nuclei in

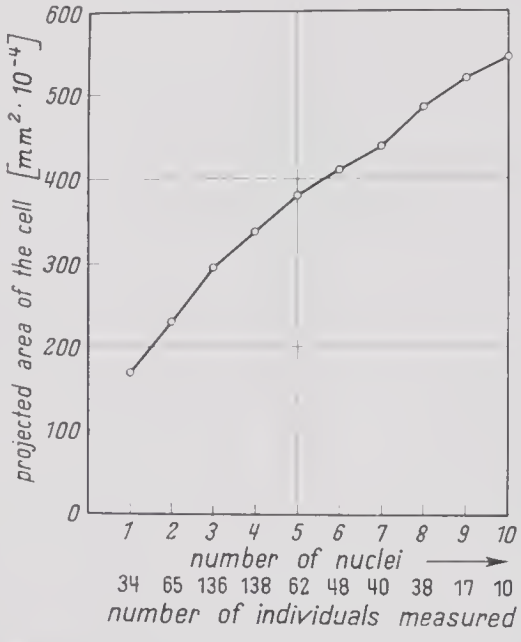

FIG. 44. *Patellina corrugata* (foraminiferan). The correlation between the number of nuclei and the ultimate size of fully grown agamonts at meiosis. The projected area of the cell is taken as a measure of the critical mass required for the initiation of meiosis. Each point represents the mean of measurements from a number of individuals as indicated under abscissa. After Grell (1959).

growing agamonts is variable, and in this case, there is a positive correlation between the number of nuclei and the critical size of the agamonts (Fig. 44): the higher the number of nuclei, the larger the agamonts must become in order to reach the critical size required for the initiation of meiosis. A given amount of cytoplasm per nucleus is evidently needed to bring about a cytoplasmic state required for inducing the nuclei to begin meiosis. In order to demonstrate experimentally that the onset of meiosis is not due to autonomous changes within the nuclei, transplantations of nuclei which normally would not

[12] This holds also for the heliozoan *Actinophrys sol*, where Straub (1951) could accelerate the "induction of meiosis" with certain substances (adrenaline, cysteine).

enter meiosis should be made into the cytoplasm of growing agamonts. It is indeed most fortunate that *Patellina corrugata* will sometimes do this experiment for us. Agamonts occassionally pick up nuclei of "Restgamenten" which found no partner at copulation and transfer them into their own cytoplasm. The nuclei of these gametes are of course haploid. Although originally highly condensed, they assume in the cytoplasm of the agamont the appearance of the normal diploid nuclei. When the latter begin meiosis, the chromosomes of the haploid nuclei undergo the cyclical changes which are characteristic of meiotic chromo-

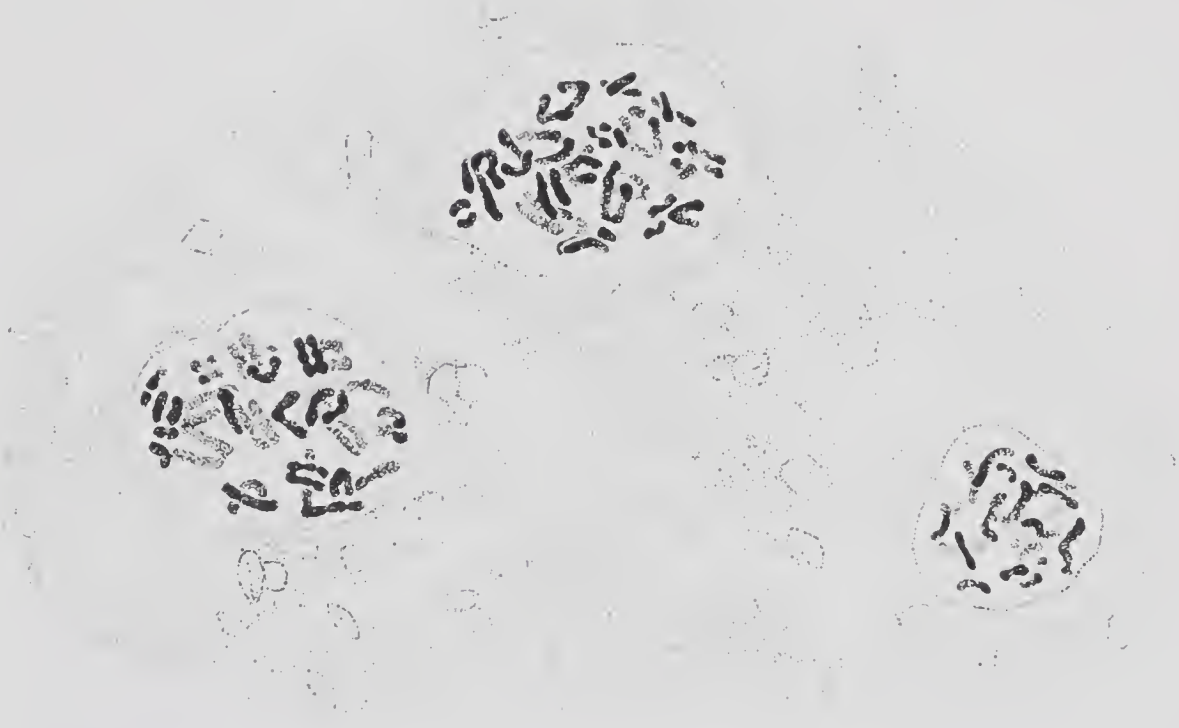

Fig. 45. *Patellina corrugata*. Three nuclei of an agamont during meiotic prophase (diakinesis). The two nuclei at left are diploid and contain bivalents, while the single nucleus at right is haploid and shows univalents only. Feulgen reaction. After Grell (1959).

somes (Fig. 45), and the haploid nuclei will even form a spindle and divide. Since the chromosomes are univalents, they are capable only of asynaptic pseudomeiosis somewhat like the somatic nuclei of heterokaryotic species although, in the latter, asynapsis is due to lack of pairing of homologs rather than to haploidy.

Although one might think of a substance whose concentration must exceed a specific value in order to induce the nuclei to react with meiosis, we do not know the nature of the cytoplasmic conditions inducing meiosis in agamonts.

A similar correlation between the number of nuclei and the critical size inducing meiosis as found in *Patellina corrugata* does not seem to hold for generative nuclei of heterokaryotic species. Meiotic agamonts of *Rotaliella roscoffensis* are all of the same size regardless of the number of generative nuclei which they contain. This supports once again the idea that only the somatic nuclei are concerned with metabolism in heterokaryotic species.

2. Gene Activation

Whenever changes of the cytoplasmic state induce nuclear division, it seems reasonable to assume that we are dealing with releasing

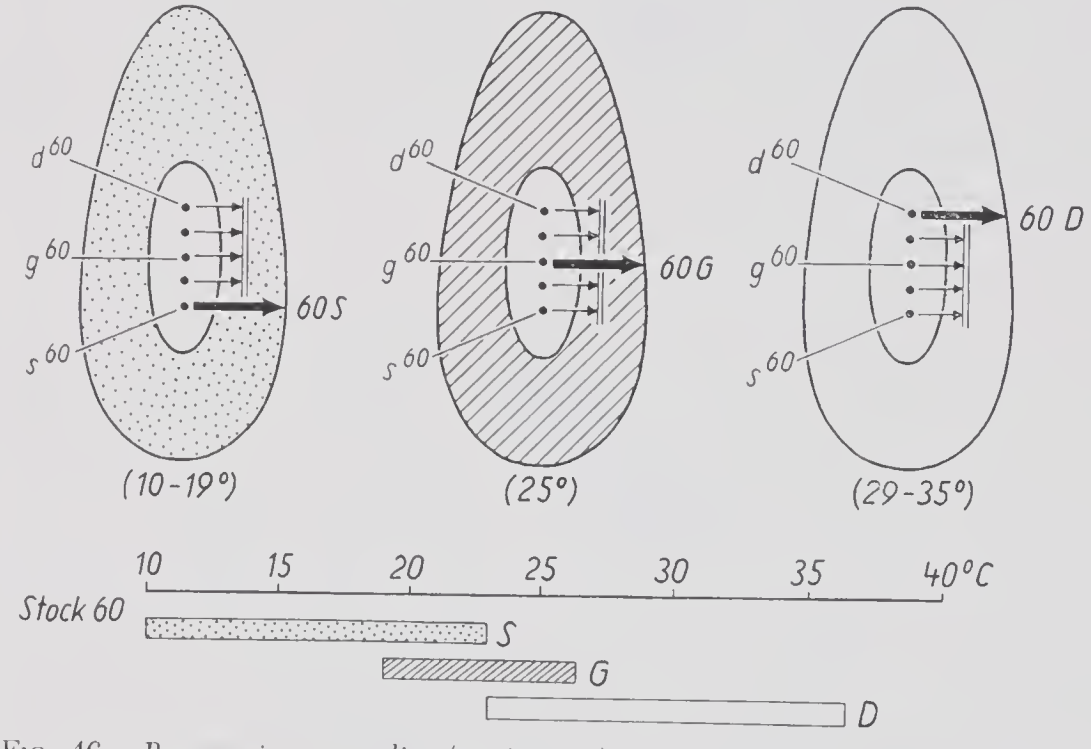

FIG. 46. *Paramecium aurelia* (variety 1). Interaction between temperature-dependent cytoplasmic states and antigen-determining genes in stock 60. The scale beneath the diagrams indicates the temperature ranges in which the corresponding cytoplasmic states are stable. After Beale (1954).

mechanisms operating via the genetic apparatus by activating certain patterns of genes. This leads to the question whether there are any more direct indications of cytoplasmic conditions leading to the activation of certain genes. The possibility of mating type determination through alternative activation of pseudoalleles within a complex locus has already been discussed (see footnote 10). In group B varieties of *Paramecium aurelia*, this activation depends upon the cytoplasm.

While these considerations are more or less hypothetical, the genetics of ciliates has yielded one definite example of the activation of certain

genes by a certain cytoplasmic state. Beale (1952), working with variety 1 of *Paramecium aurelia*, found the genes for the ciliary antigens S, G, and D to be active only within certain temperature ranges (Fig. 46). However, temperature is not acting directly on the genetic apparatus; rather, it causes a cytoplasmic state which is preserved after the treatment and is handed on through a succession of cell divisions. Beale could demonstrate this by crossing strains whose antigens, although formed at the same temperature, can be distinguished serologically. Since these differences depend on alleles, heterozygous clones could be obtained from exconjugants in which the antigens characterizing both parental strains were formed. It was shown that the allele brought in by the cross can be activated if the state of the cytoplasm is such as to induce activity of the allele brought in by the cytoplasmic parent.

In the reviewer's opinion, this represents the most impressive example of the interplay of external factors, cytoplasmic conditions, and genetic apparatus of the cells nucleus from the wealth of results which ciliate genetics has so far yielded.

VI. Movements of Nuclei

A discussion of relationships between nucleus and cytoplasm in Protozoa would be incomplete if attention were not briefly focused on the movements of nuclei in certain phases of the developmental cycle. We are concerned here not with accidental changes of position within the cell, but with *specific movements* executed in connection with certain reproductive processes. Although they are no doubt due to cytoplasmic streaming, we have to assume the existence of nuclear control. In heterokaryotic foraminiferans there are indications that nuclear movement depends on the state of determination since somatic nuclei always leave the initial chamber earlier than generative ones. We do not know the nature of these interactions between nucleus and cytoplasm. What factors regulate the even distribution of generative nuclei before meiosis in foraminiferans, or, for instance, the migration of the male pronucleus to the female one in the polymastigote flagellates of *Cryptocercus*? How is it that the nuclei of gametes find each other? An observation by Cleveland (1957a) seems to indicate that this is not necessarily connected with the sexual differentiation of the pronuclei. If a female gamete of *Trichonympha* copulates with two male gametes, the nuclei of the male gametes may fuse with each other (Fig. 47). What factors are responsible for the migration of one of the two pronuclei of ciliates into the partner while the other pronucleus remains stationary? Schwartz (1952) believes that the migratory pronucleus might simply be pushed into the partner

by spindle elongation at the last progamic division. However, there are cases where the division figure is parallel to the border between conjugants, nuclear migration occurring later and entirely independent of this (Kormos and Kormos, 1960). Nor is the tendency to migrate dependent upon the presence of normal haploid pronuclei, as Chen

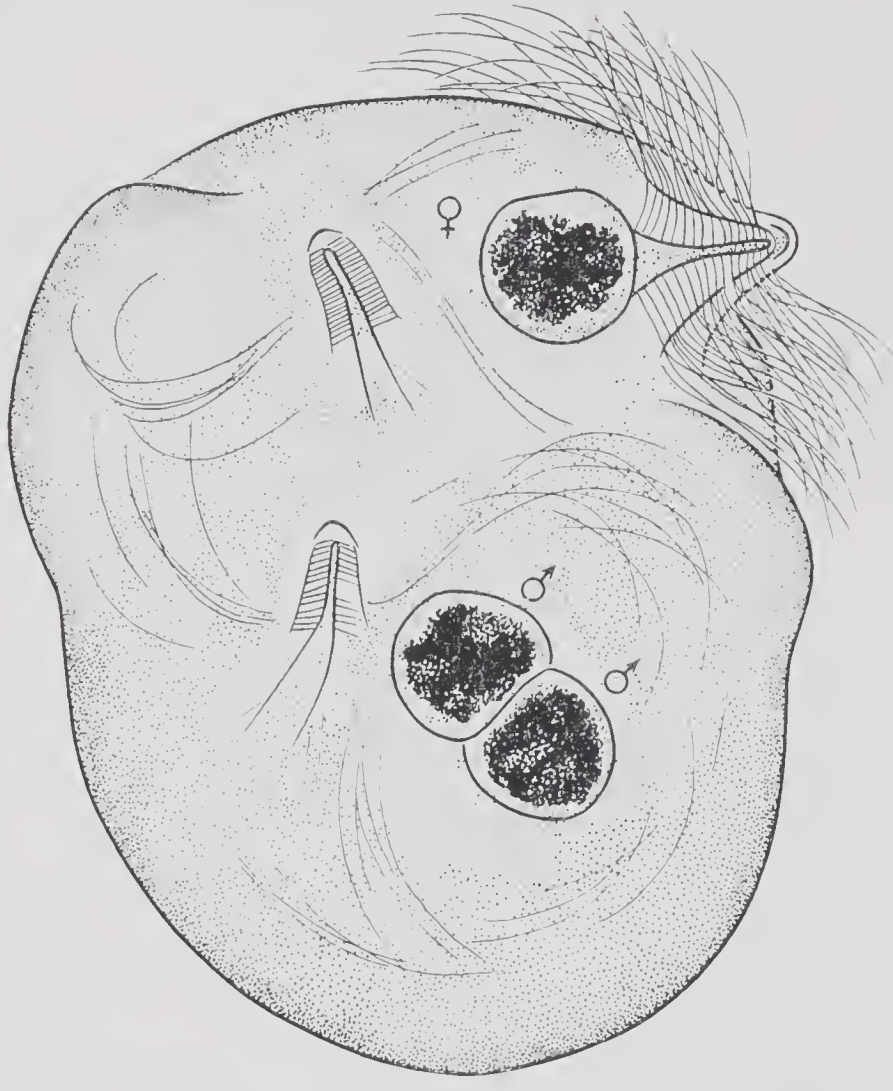

FIG. 47. *Trichonympha* (polymastigote flagellate from the wood-feeding roach *Cryptocercus punctulatus*). Exceptional case of fertilization, involving the fusion of two male gametes with a single female. After loss of extranuclear organelles the two male pronuclei are fusing with each other, but not with the female pronucleus. After Cleveland (1957a).

(1946) could show in an analysis of nuclear events at conjugation of different varieties of *Paramecium bursaria*. Russian and American conjugants proved incompatible. Although mating commenced, chromosomal clumping at the first progamic division prevented the formation of pronuclei; cytoplasmic streaming set in, although an actual exchange of nuclei could not occur.

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The following *films* pertaining to the material presented in this review can be obtained through the Institut für den wissenschaftlichen Film in Göttingen, Nonnenstieg 72, Federal Republic of Germany:

E 407. *Paramoeba eilhardi* (Amoebina). Fortbewegung.
C 829. Morphologie der Radiolarien.
C 801. Morphologie der Foraminiferen.
C 802. Fortpflanzung der Foraminiferen.
C 684. Die Entwicklung von *Eucoccidium dinophili.*
C 836. Mittelmeerplankton. Protozoen.

CHAPTER 2

The Cytoplasm of Protozoa

By WILLIAM TRAGER

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I. Introduction

The protozoan cell must be capable of performing all those functions which in multicellular organisms are distributed among numerous cells of different kinds. Hence it is not surprising that a single protozoan cell may exhibit a diversity of structures and functions rarely encountered in any one cell of a multicellular plant or animal. Yet the fundamental plan of organization of the protozoan cell is the same as that of the metazoan cell—a fact long apparent but recently made strikingly clear through studies on fine structure as seen with the electron microscope (Grimstone, 1961). The nucleus of the protozoan cell—its structure, its chromosomes, its modes of division, its functions in heredity and morphogenesis—have been treated by Grell in Chapter

1 of this volume. The present chapter will consider the protozoan cytoplasm and its organelles both as valuable materials for the study of structures and functions common to all cells, and as examples of cellular specializations not known elsewhere in the living world (Fig. 1).

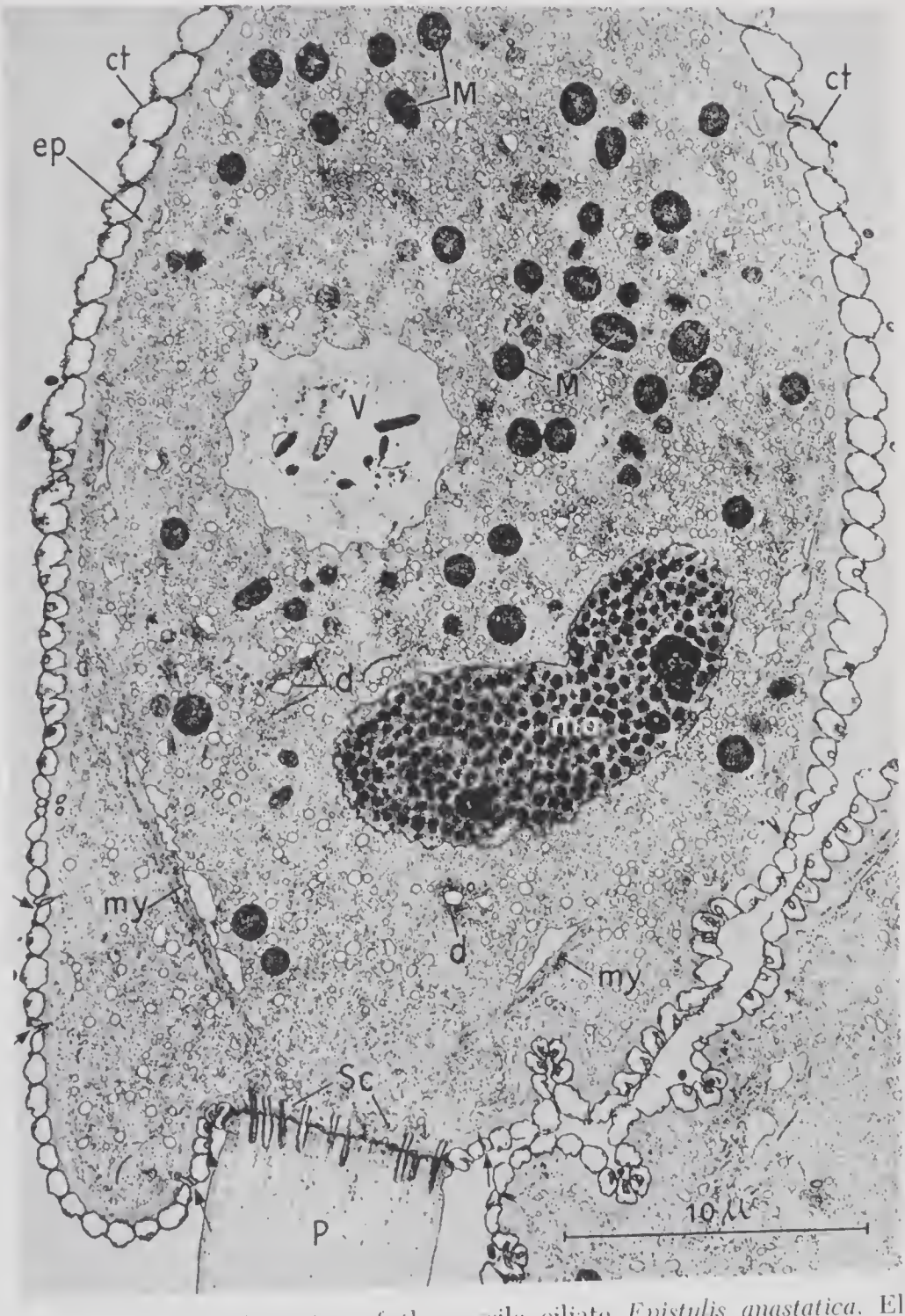


FIG. 1. Longitudinal section of the sessile ciliate *Epistylis anastatica*. Electron micrograph. *Ct*, cuticle; *Ep*, epiplasm. Arrows indicate pores in cuticle. *M*, mitochondria; *d*, dictyosomes; *V*, food vacuole; *my*, myonemes; *Sc*, scopula; *P*, peduncle; *Ma*, macronucleus. From Fauré-Fremiet *et al.* (1962).

II. The Surface

A. *General Structure and Protective Functions*

The surface of a cell is much more than a cellophane-like bag serving to separate the living contents from the nonliving medium; it is a region of intense biological activity associated with diverse physiologieal needs. Since a protozoan cell is also an organism, its surface must be exeeptionally versatile: it must serve for protection; for the mediation of environmental factors, as in osmoregulation; for the detection and uptake of nutrients; for the discharge of waste products; for locomotion; for sexual union; for the release of aggressive structures or substances. Although one can describe in a qualitative way what happens during some of these processes, the biochemical and biophysical events underlying them are not understood, a not surprising situation in view of the diffieulties encountered in attempts to understand the structure and function of such a relatively simple cell surface as that of the erythrocyte (see E. Ponder, Chapter 1 in Volume II of this treatise).

All protozoa have at least one unit plasma membrane 70–100 A. thick (Grimstone, 1961). Many species have two such membranes side by side at the cell surface, and still others have three. One pattern is well illustrated in the freeliving ciliate *Epistylis* (Fauré-Fremiet *et al.*, 1962) where there is one external membrane having beneath it a system of cireular canals bound by a second membrane, giving the equivalent of three membranes with spaees between the middle and innermost membranes (Fig. 2). More usual among the free-living ciliates is a set of three membranes, the innermost one closely applied throughout to the eetoplasm, whereas the two outer membranes form a pellicle thrown into folds or ridges (Pitelka, 1961a), forming a structure with an average thickness of about 250 A. At the base of each fold the innermost membrane is eontiguous to the middle membrane but elsewhere these two membranes are separated by a considerable space. This same arrangement is found in some free-living flagellates (Roth, 1959) and in gregarines (Fig. 3) (Kümmel, 1958; Beams *et al.*, 1959). The added structural strength of systems sueh as these, as compared with smooth double or triple membranes, is obvious. Possibly serving the same purpose are subpellicular fibers present in many flagellates both free living (Roth, 1959) and parasitic (Lofgren, 1950) (Fig. 4).

Free-living amebae have a relatively thick (up to 0.2 μ) external coating of polysaccharide (Pappas, 1959; Greider *et al.*, 1958). In electron micrographs this often appears as a layer of frayed, short fibrils

or filaments. Similar material has been demonstrated on the surface of the hypermastigote flagellate *Trichonympha* (Grimstone, 1961) and of gregarines (Beams *et al.*, 1959). Its functions may be not unlike those of the slime secreted by larger organisms, as snails, which move in close contact with a substrate.

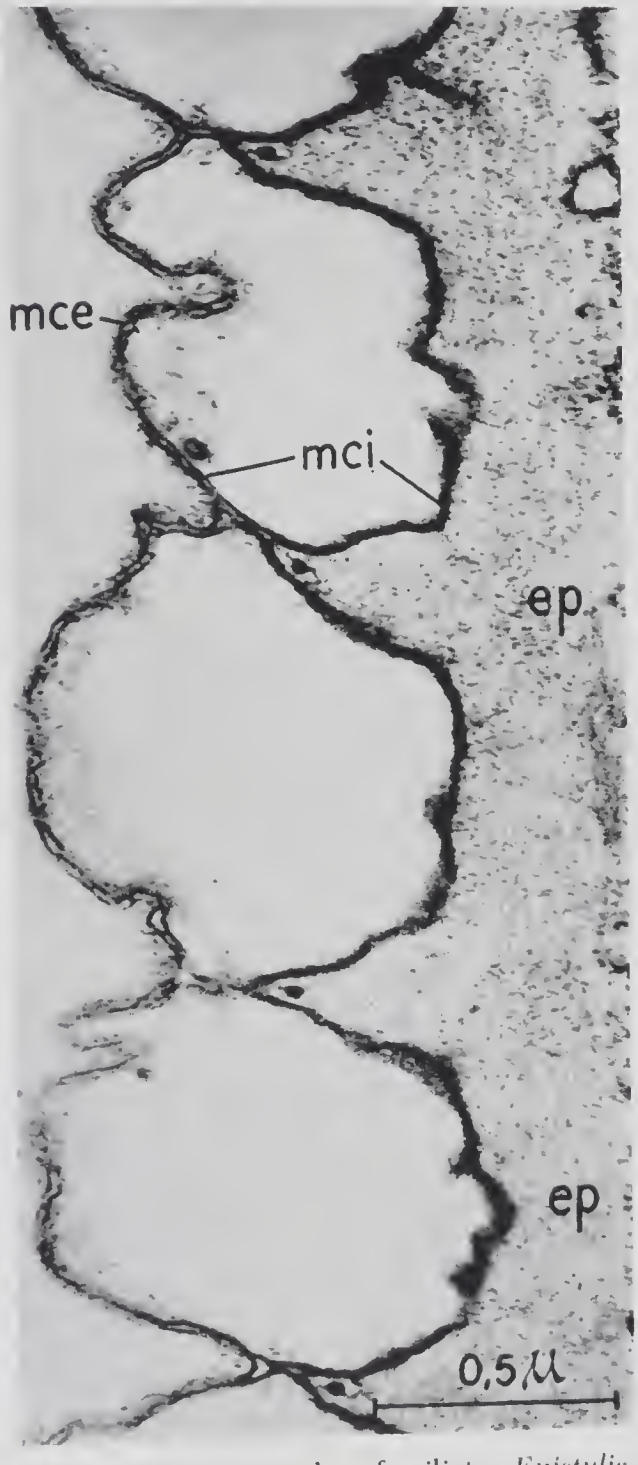


FIG. 2. Transverse section of cuticle of ciliate *Epistylis anastatica* showing internal (*mci*) and external (*mce*) membranes and epiplasm (*ep*). Electron micrograph. From Fauré-Fremiet *et al.* (1962).

The formation of protective shells or cases is a common phenomenon among protozoa (Manwell, 1961; Grassé, 1953). The shell may be formed from foreign particles picked up and cemented around the body, or, more often, it is secreted from the cell surface. It may then consist of calcium carbonate or silica, of cellulose or protein. Always the form of the shell is a highly specific character. Virtually nothing is known concerning the mechanism of formation of such shells, or

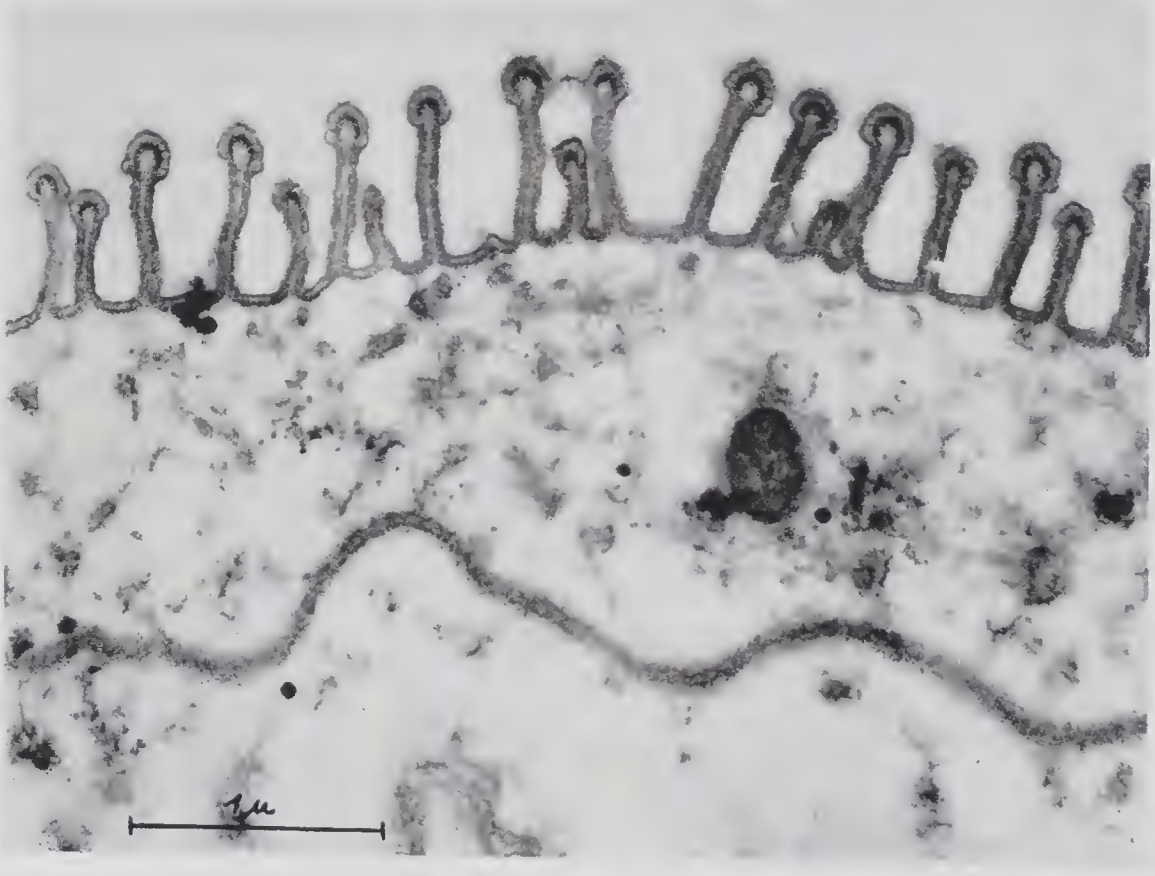


FIG. 3. Section through surface of gregarine *Beloides* showing pellicular ridges. Electron micrograph. From Kümmel (1958).

even of the protective wall which appears during cyst formation. The complexity which these structures may achieve is illustrated in the beautiful shells of the foraminifera, in the cellulose thecae of the dinoflagellates, and in the protein and acid mucopolysaccharide shells of rhizopods such as *Gromia oviformis* (Hedley and Bertand, 1962). In *Gromia*, which may reach a diameter of 5 mm., the shell has a stratified structure and is perforated by radial canals. On the inner surface of the shell wall are up to ten delicate honeycomb-like membranes. One may surmise the existence of special secretory organelles associated with shell formation, perhaps resembling the secretary am-

pule concerned with formation of a cement for temporary attachment in the ciliate *Trichopus* (Fauré-Fremiet, 1957).

The resistant spore of the microsporidia provides an example of extreme cytoplasmic specialization, though it is not clear whether all the structures are derived from the surface membranes. Microsporidia are best known as intracellular parasites of insects, though recently a species has been described within the cells of an ascites tumor of

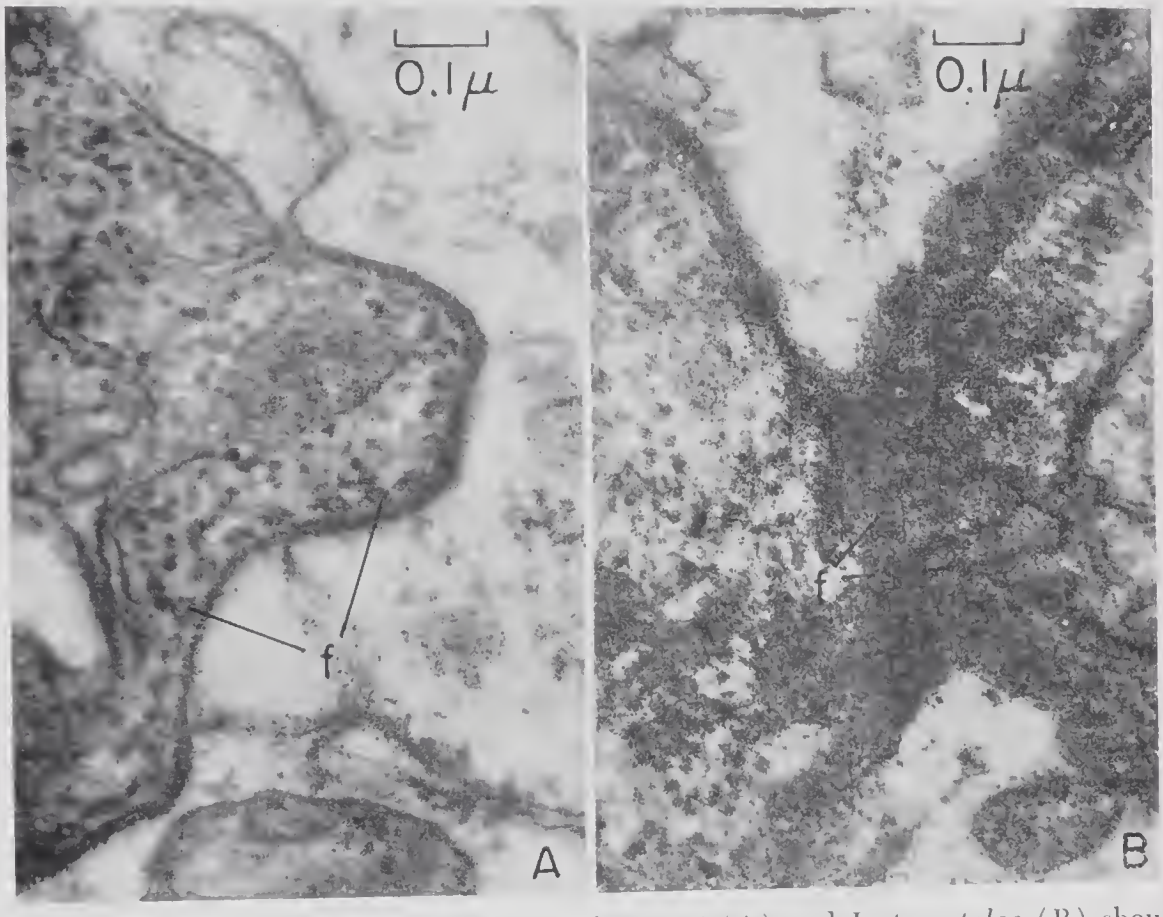


FIG. 4. Sections through *Leishmania donovani* (A) and *L. tarentolae* (B) showing subpellicular fibers (*f*) in cross section (A) and tangential section (B). Electron micrographs. Rudzinska and Trager, unpublished.

mice (Nelson, 1962). After a period of multiplication in the host cell the parasite forms a spore having a thick wall, and within this a structure called a polar capsule and a tightly coiled springlike polar filament (Kudo and Daniels, 1963). Only a small portion of the interior of the spore is occupied by a mass of nucleated cytoplasm. Under appropriate conditions (as after ingestion by a suitable new host) the spore hatches with the violent extrusion of the polar filament bearing at its tip the cytoplasmic mass, which now appears as a uninucleate amebula (Dissanaike and Canning, 1957).

B. Ingestion of Nutrients

Many well-known free-living protozoa feed in nature on particulate matter. Amebae engulf particles by phagocytosis, whereas most ciliates have a specialized mouth through which particles are ingested. Both exercise some selection with respect to the materials taken up or discarded, so that the surface must have a mechanism for recognizing different classes of substances. Although some species of amebae may ingest at any region of the body, most species have a differentiated tail region or uroid (Hollande, 1942), which seems to have an exceptionally sticky surface and to be specially concerned with the gathering and uptake of food. The process of phagocytosis is intimately related to ameboid movement in general and is equally poorly understood.

The mouth of ciliates is an elaborate organ (Fig. 5) (Ehret and Powers, 1959), but once a food particle has been ingested by it and passed through the gullet, it enters the cytoplasm just as do the particles ingested by amebae. In either case the food is contained in a food vacuole, a structure bound by a membrane, into which enzymes are secreted and which shows analogies to the lysosomes of the cells of higher animals (Mast, 1942; Muller and Törö, 1962). There is now evidence from electron microscopy that the further processes of food absorption are similar in both amebae and ciliates. In the large ameba *Pelomyxa* the membrane of the food vacuole, formed from the plasmalemma with its coat of filamentous material, first becomes smooth and straight. Then its surface area is greatly increased by the elaboration of numerous folds and fingerlike protrusions, all during the first hour after formation of the vacuole (Roth, 1960). At this time the vacuolar volume is still increasing, so that fluid is evidently being transferred into the vacuole; presumably this is related to the digestion of the food particle. Later the vacuolar membrane changes again with the appearance of numerous vesicles 0.2–0.5 μ in diameter on its cytoplasmic side. The content of the vesicles has the same appearance as material lying within the food vacuole next to the membrane. Evidently the digested food in solution is taken up from the vacuole by micropinocytosis.

In the ciliate *Paramecium* the membrane of older food vacuoles is likewise surrounded by small vesicles (Fig. 6a) formed by pinocytosis (Jurand, 1961). Furthermore, such small vesicles are especially abundant around the deepest part of the buccal cavity (Fig. 6b). It would appear that in ciliates, as in amebae, nutrients in solution may be taken up by a process of vacuole formation, either around a food vacuole

FIG. 5. Section through *Paramecium bursaria* at level of buccal cavity, looking anteriorly. Electron micrograph. Directly above the macronucleus are 8 columns of cilia constituting the ventral and dorsal peniculi; clockwise from these are the 4 columns of cilia making up the quadrulus; above that is the ribbed wall. From Ehret and Powers (1957).

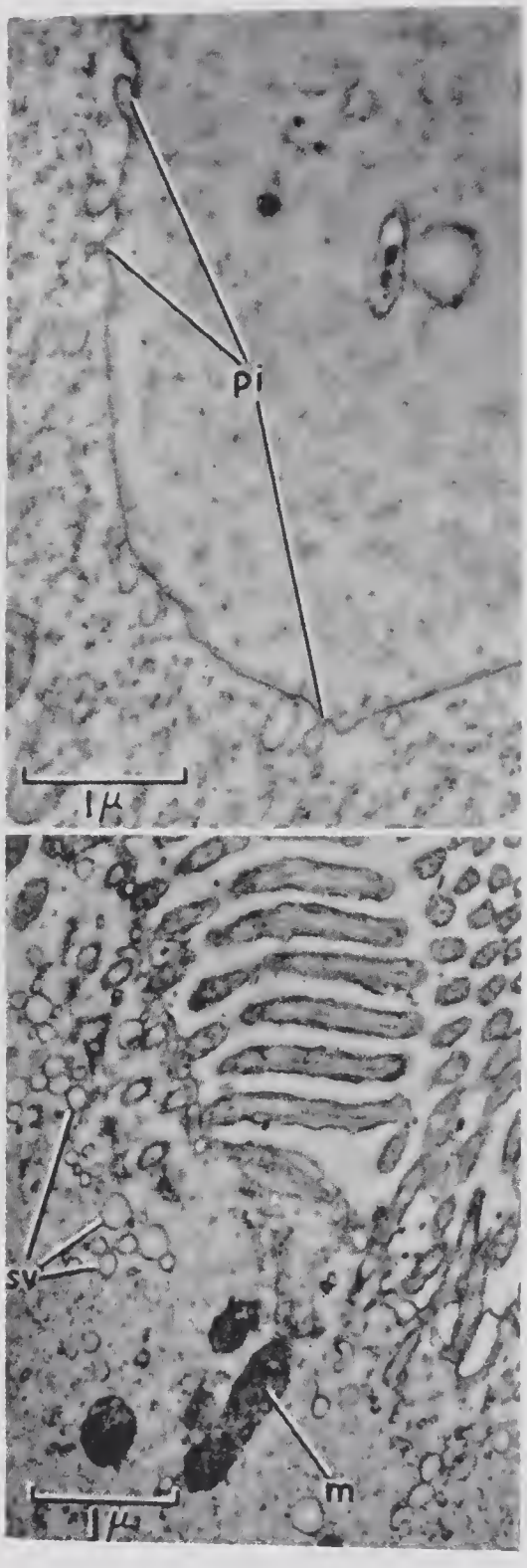


FIG. 6. Electron micrographs of sections through *Paramecium aurelia*. Upper fig., an older food vacuole with protuberance (*pi*) suggesting pinocytosis. Lower fig., deeper part of buccal cavity surrounded by numerous secondary vacuoles (*SV*). From Jurand (1961).

within which solid food has been digested, or directly from the surrounding meduim.

This mechanism of fluid ingestion, first noted by Edwards (1925), has been seen in electron micrographs of many different cells and has been studied in much detail in amebae (Holter and Marshall, 1954; Schumaker, 1958; Holter, 1959). Charged solutes stimulate the process whereas uncharged ones do not. Possibly charged molecules directly

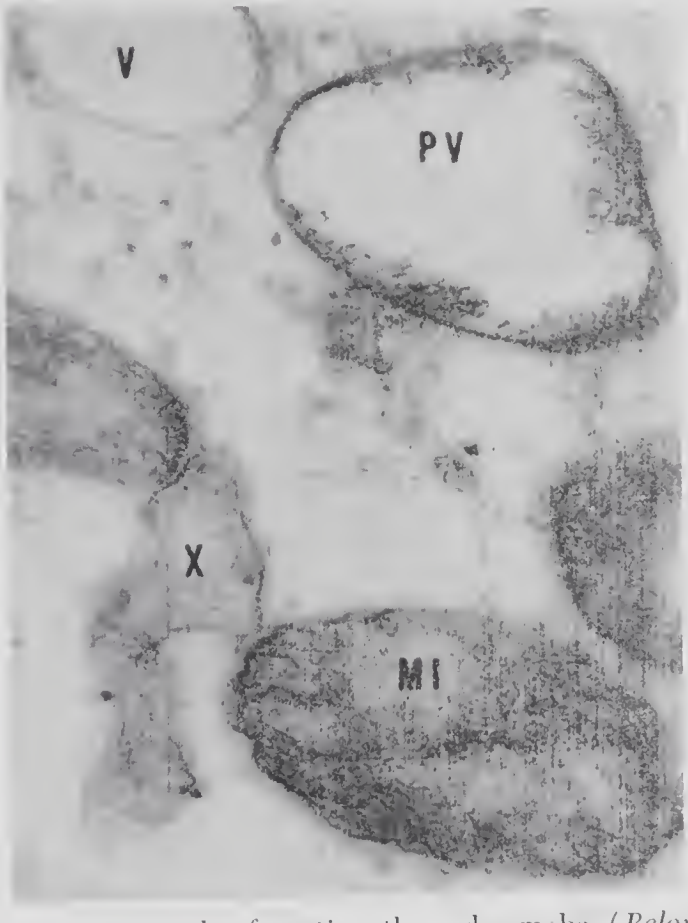


Fig. 7. Electron micrograph of section through ameba (*Pelomyxa carolinensis*) to show recently formed pinocytic vesicle (*PV*) with filamentous fringe. *V*, cytoplasmic vesicle; *MI*, mitochondrion; *X*, body of unknown nature. Magnification: × 32,000. From Roth, (1960).

affect the surface tension of the plasmalemma; or perhaps when absorbed they change the internal bonding and hence the structural rigidity of the plasmalemma. In living amebae, the infolded plasmalemma has been seen to be freed from the underlying ectoplasmic gel in a series of jerks (Brandt, 1958). When the pinocytic vesicle is first formed its origin from the plasmalemma is clearly indicated in electron micrographs by the presence on its inner surface of the same frayed filamentous material which characterizes the outer surface of the ameba (Fig. 7) (Roth, 1960). Vesicles deeper in the cytoplasm

lack this "fringe" although their membrane still resembles the plasma membrane in being stainable with phosphotungstie aeid. Sinee vesieles are also present having a generally similar appearanee but with a membrane not stainable by phosphotungstic acid, it seems reasonable to suppose that the membrane gradually changes its properties to permit penetration into the cytoplasm of materials contained in it.

This fundamental unity in mechanism of nutrient uptake in protozoa as different as amebae and eiliates, and the presenee of the same mechanism in various types of eells in metazoa, suggests that osmotrophy, implying uptake of nutrients by penetration direetly through the cell membrane, may be of minor importanee or may not oeeur at all. This possibility is strengthened by observations with two kinds of parasitic protozoa. When eulture forms of the frog trypanosome *Trypanosoma mega* were suspended in a medium eontaining ferritin and subsequently sectioned and examined by electron mieroseopy, it was found that the ferritin mieelles adhered to the pellicle at only one well-defined region of the body. Only at this region, representing a funetional cytostome, were the ferritin micelles ingested as elusters and held in pinocytie vacuoles (Steinert and Novikoff, 1960). Malaria parasites, whieh grow within erythroeytes, have long been known to digest hemoglobin, and there was some question as to how this large molecule penetrated into the eell, sinee the hemozoin remaining after its digestion was within the body of the parasite. Eleetron mierographs of sections of erythroeytes infected with any of several species of malaria parasites showed elearly that the eytoplasm of the host red cell is ingested into food vaeuoles by a proeess of intraeellular phagotrophy or pinocytosis (Fig. 8) (Rudzinska and Trager, 1957, 1959). In the malaria parasites, as digestion of hemoglobin proeeeds within the food vacuole, no visible ehange oeeurs in the vaeuolar membrane; this remains as a double membrane identieal with that separating the parasite from its host eell. Funetionally, however, the membrane must be quite different, since within it hemoglobin disappears and hemozoin is formed, whereas no sueh ehanges oeeur in host eell cytoplasm lying adjaeent to but outside the body of the parasite. This same method of food intake takes plaee in another kind of intraerythroeytie parasite, *Babesia* (Rudzinska and Trager, 1962), but no indieation of it has so far been seen in parasites living in host eells other than erythrocytes [*Toxoplasma*, for example (Gavin *et al.*, 1962)]. Perhaps some of these parasites will remain as true osmotrophs.

Some recent experimental studies would indicate that the free-living eiliate *Tetrahymena pyriformis* can obtain most of its nutrients

by osmotrophy (Seaman, 1961). This ciliate grown in a chemically defined medium did not ingest trypan blue but was readily permeated by 3,5-dinitrotyrosine, which presumably entered through the cell wall. Even in a peptone-yeast medium, which induced extensive food vacuole formation and uptake of trypan blue, the extent of uptake of the dye indicated that phagotrophy could not adequately supply the nutritional

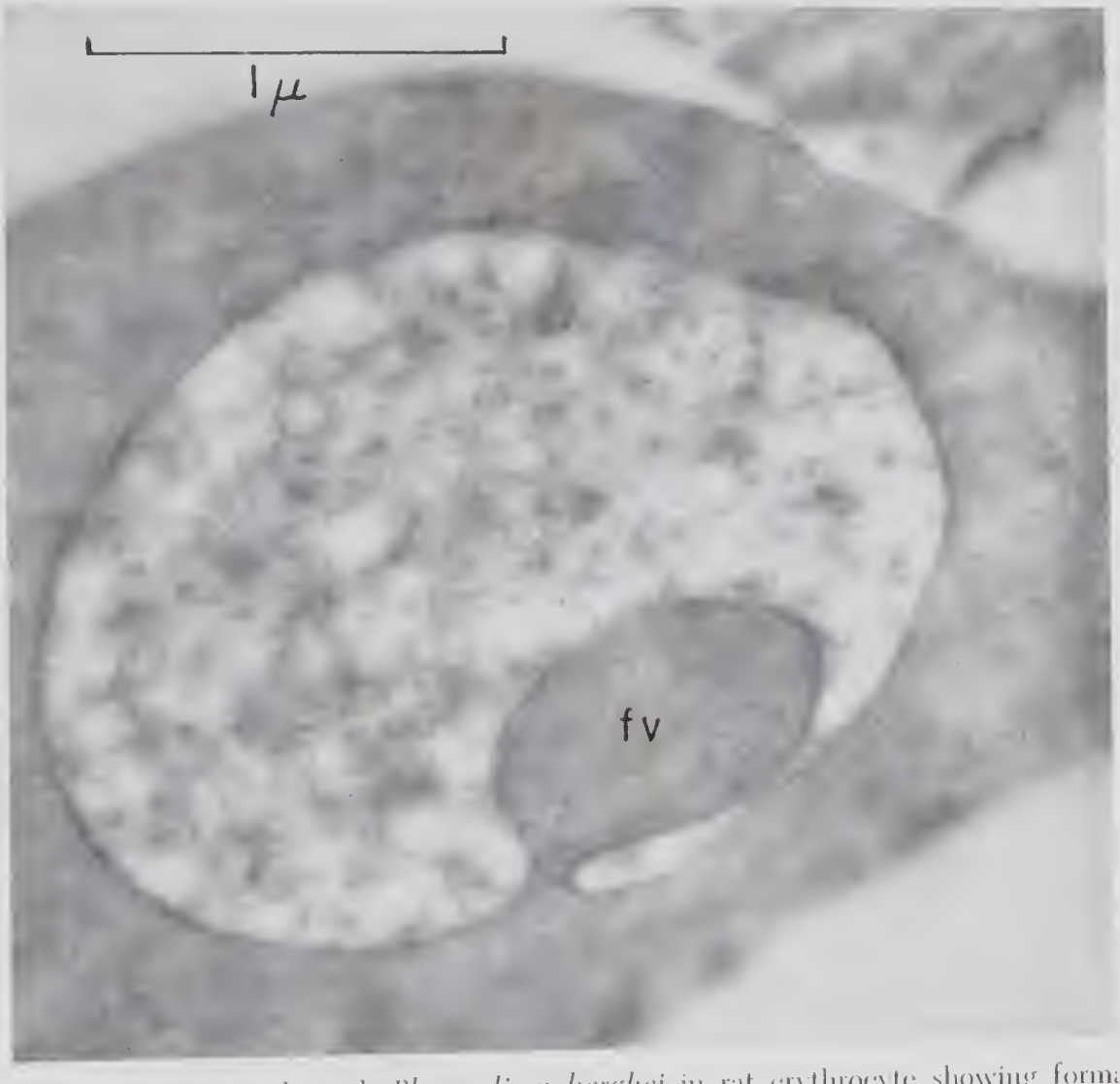


FIG. 8. Section through *Plasmodium berghei* in rat erythrocyte showing formation of food vacuole (*fv*). Electron micrograph. From Rudzinska and Trager (1959).

demands of the organism. This conclusion, however, should be carefully reexamined in light of the facts that *Tetrahymena* has a rigid pellicle and a well-developed mouth, that it feeds in nature on bacteria and other particles, and that pinocytosis is such a widespread and effective means for the cellular uptake of fluid and solutes. An experiment of the type done by Steinert and Novikoff with ferritin and *Trypanosoma mega* might be particularly instructive. Certainly the

problem of osmotrophy and its exact mechanism is of great interest and significance in relation to the nutrition of protozoa and other aquatic invertebrates.

C. Osmoregulation

Whatever the detailed mechanism of fluid uptake by protozoa may be, there is no doubt that protozoa living in fresh water must be able to maintain an internal osmotic pressure higher than that of the medium. All such protozoa (but not most parasitic protozoa or marine forms) have a conspicuous contractile vacuole, an organelle whose main function has long been considered to be the elimination of excess water from the cell (Kitching, 1952b). That this is indeed the sole function of the contractile vacuole has been conclusively shown for the phytoflagellate *Chlamydomonas moewusii* (Guillard, 1960). An X-ray-induced mutant of this species, lacking contractile vacuoles, was observed to survive only in media having an osmotic pressure over 1.5 atmospheres provided by solutes to which the cell was relatively impermeable, such as NaCl or sucrose. In a medium containing such compounds and having an osmotic pressure over 2.1 atmospheres the mutant (S-16) grew as well as the parent strain. There was no growth in isosmotic solutions of rapidly penetrating solutes such as ethylene glycol, whereas there was intermediate growth with urea or glycerol, which penetrate at intermediate speeds. S-16 cells would ultimately burst in solutions of low osmotic pressure. When placed in solutions permitting growth of S-16, the wild strain did not show contractile vacuoles, whereas it did show them in isosmotic solutions of ethylene glycol. Hence S-16 differs from its parent strain in having lost the ability to form contractile vacuoles; these are essential for the elimination of excess water when the organism is in a medium of low osmotic pressure. It has in effect become an obligate brackish water organism.

Contractile vacuoles differ widely in complexity of organization in different groups of protozoa. In some they appear to form and discharge at almost any region of the cell surface; in others they are always in the same position and are associated with a permanent opening in the cell surface, the contractile vacuole pore. The vacuole may be a sac lined by a simple membrane or it may be part of a complex nephridial system, as in *Paramecium* (Schneider, 1960). In this organism, electron microscopy of thin sections has revealed a network of fine, branching nephridial tubules of 200 A. diameter surrounding the nephridial canals and evidently derived from the endoplasmic reticulum. During diastole of the radial canals the nephridial

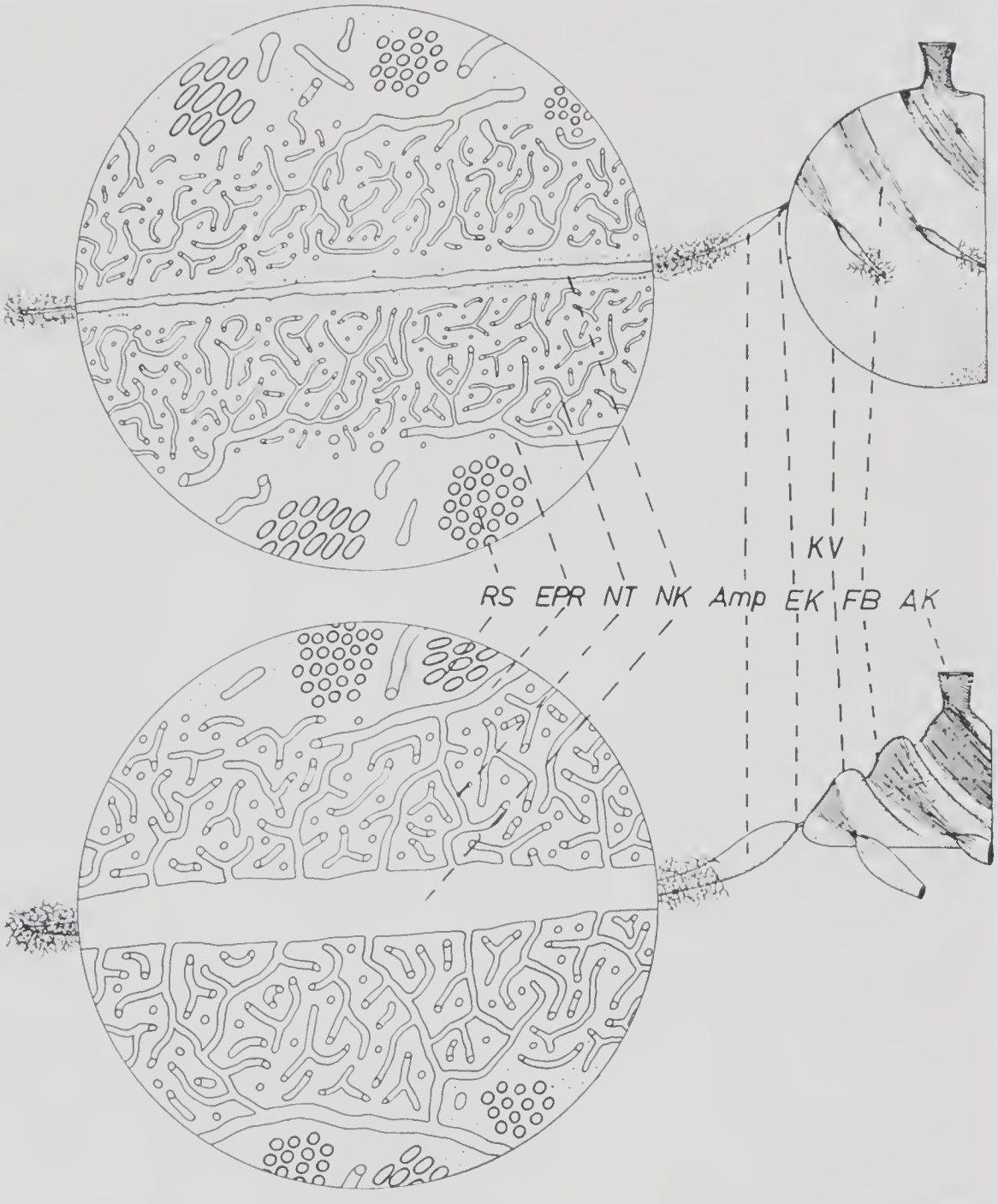


FIG. 9. Schematic representation of the nephridial system of *Paramecium* as revealed by electron microscopy. Upper figure: radial canals in systole, contractile vacuole in diastole. Lower figure: radial canals in diastole, contractile vacuole in systole. *EPR*, Tube-forming membranes of endoplasmic reticulum, leading to the nephridial tubules (*NT*). These open into the nephridial canal (*NK*) during its diastole (lower) but are shut off from it during its systole (upper). The nephridial canal leads to the ampulla (*Amp*), and this via the injection canal (*EK*) to the contractile vacuole (*KV*). *FB*, Fibrillar elements; *RS*, bundle of tube-shaped structures; *AK*, canal to excretory pore. From Schneider (1960).

tubules open into the nephridial canals, but this connection is closed during systole. Surrounding the network of nephridial tubules are bundles of larger tubular structures 500 A. in diameter of unknown function. The membrane of the terminal nephridial canal continues uninterrupted into the radial ampulla, the injection canal connecting

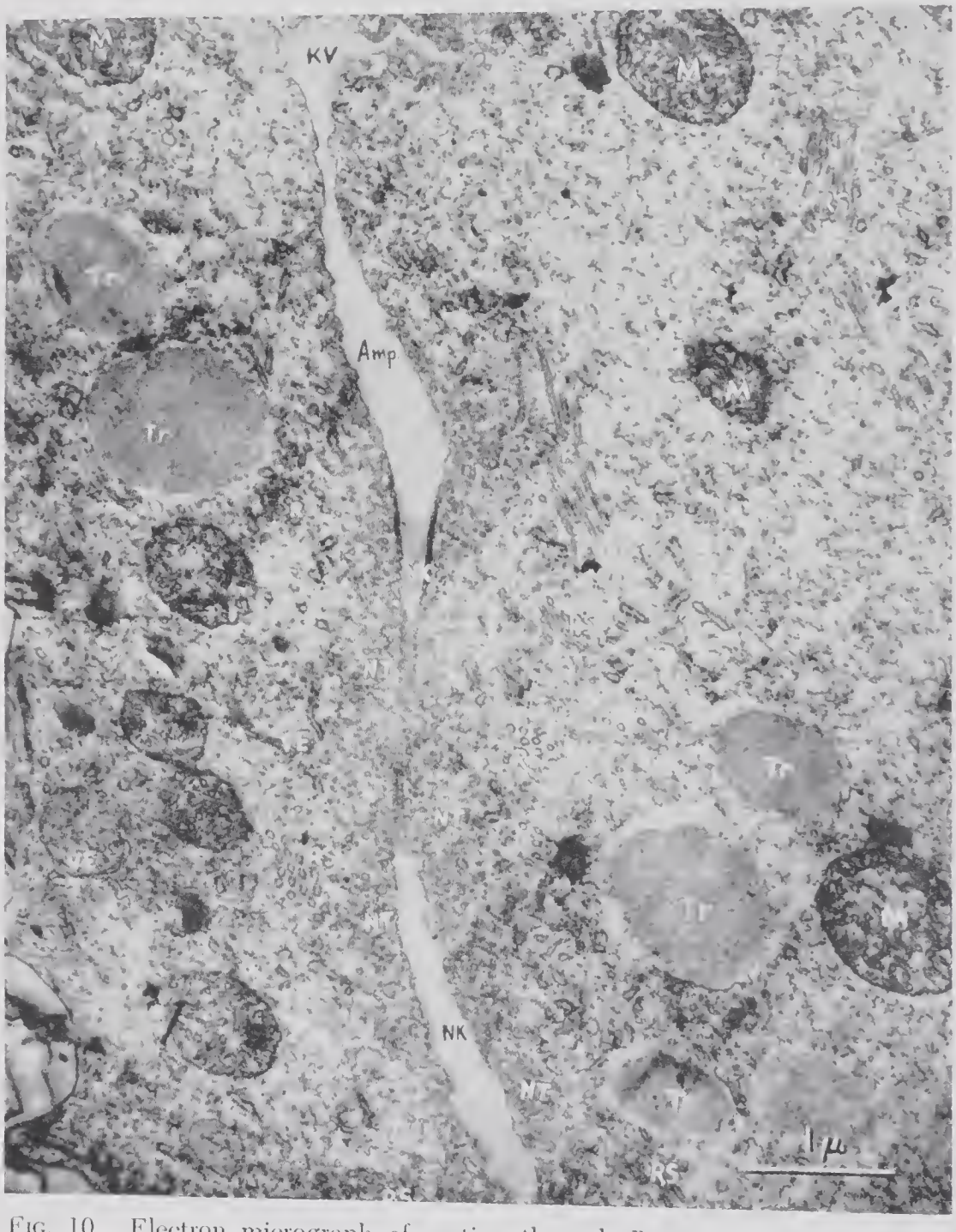


FIG. 10. Electron micrograph of section through *Paramecium*. Radial canal in transition from diastole to systole. Contractile vacuole (*KV*) in systole. *EK*, Injection canal; *Amp*, ampulla; *NK*, nephridial canal; *NT*, nephridial tubules; *RS*, bundle of tube-like elements; *Tr*, trichocysts; *M*, mitochondria; *VE*, vesicular elements of endoplasmic reticulum. From Schneider (1960).

this to the contractile vacuole, and the vacuole itself (Figs. 9–11). The ampulla, the injection canal, and the vacuole are surrounded by a spiral envelope composed of bundles of fibrillar elements, presumably contractile. The contractile vacuole during its diastole is separated from

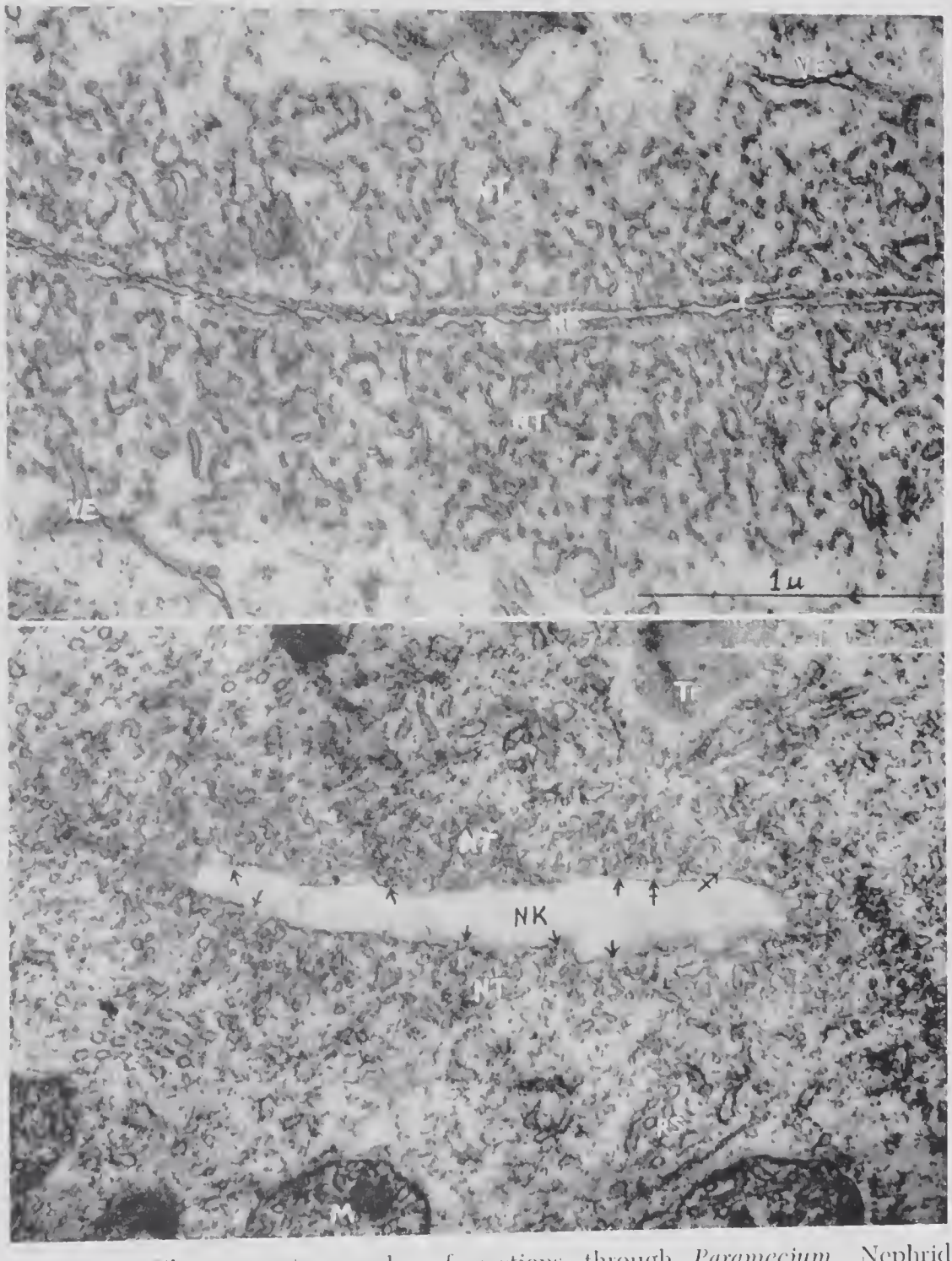


FIG. 11. Electron micrographs of sections through *Paramecium*. Nephridial canal (*NK*) in systole (above) with nephridial tubules (*NT*) shut off from it; in diastole (below) showing nephridial tubules opening into it (↑). From Schneider (1960).

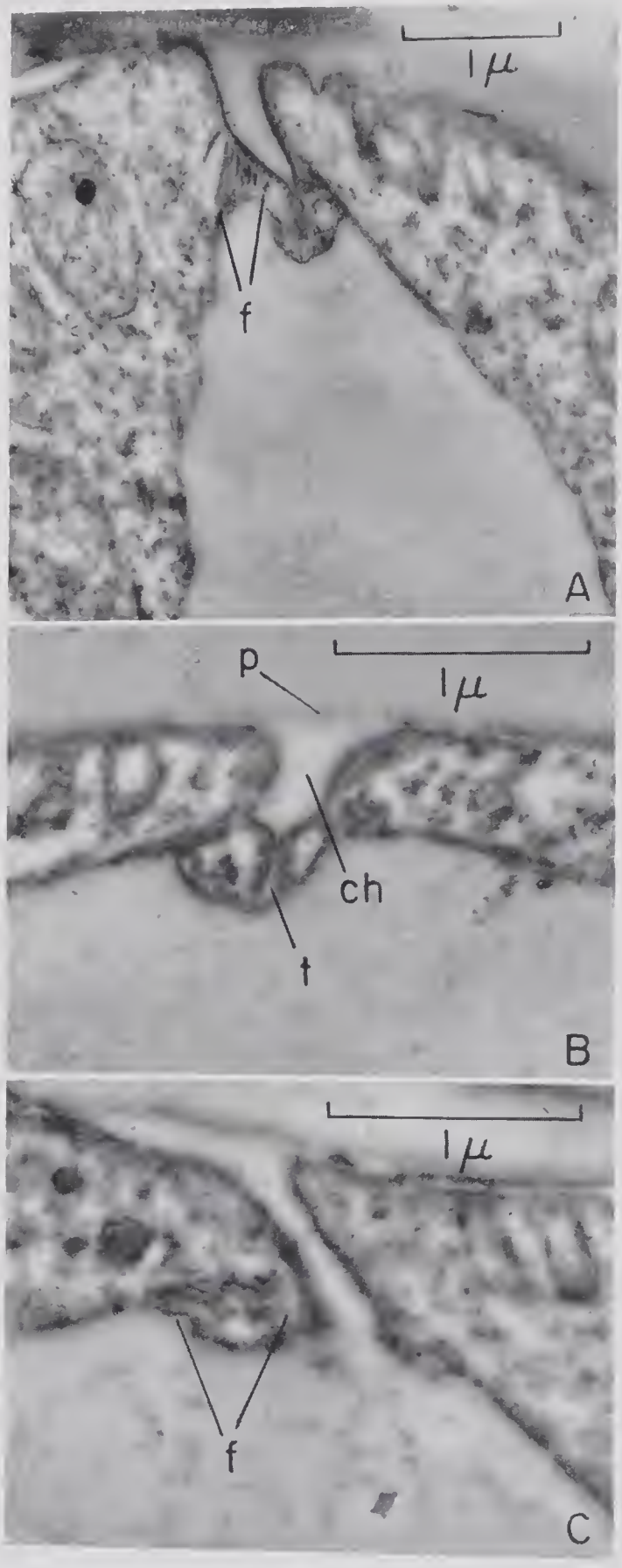


FIG. 12. Sections through the contractile vacuole of *Tokophrya infusionum* at the region of the excretory canal showing: (A) canal closed with fibrils (*f*); (B) canal closed showing pore (*p*), channel (*ch*), and tubule (*t*); (C) canal and tubule open. Electron micrographs. From Rudzinska (1958).

the canal of the excretory pore by a membrane which, it is assumed, is broken at systole. Hence, in this system fluid enters from the network of nephridial tubules into the nephridial canals, which gradually expand in diameter. These then contract in systole, at which time the openings from the tubules are closed and fluid is forced into the ampullae, which enter diastole, their connection to the injection canal being closed and the vacuole itself being in systole at this time. Upon the opening of the injection canals the ampullae empty into the contractile vacuole, which now fills with fluid in its diastole. Finally the injection canals are again closed as the fibrillar elements surrounding the vacuole contract, forcing a break in the membrane separating the vacuole from the excretory canal and expelling the fluid to the outside of the cell.

Only slightly less complex is the situation in the suctorian *Tokophrya* (Rudzinska, 1958). Although a nephridial system has not been seen in this organism, the contractile vacuole opens to the outside by means of a highly specialized valvular structure, whose opening and closing are presumably controlled by contractile fibrils (Fig. 12).

D. The Capture of Food; Aggressive and Protective Organelles

We have already seen that ingestion of food by phagotrophy and by pinocytosis, far from being a nonspecific process, involves specific responses to particular kinds of materials. This specificity is accentuated whenever the organism, rather than being a scavenger or bacteria-feeder, is an active predator and feeds on other animals, generally other protozoa. Space will permit the consideration here only of the structure and function of tentacles, in part derived from the surface, and of trichocysts, which are discharged from the cell surface.

The tentacles of the suctoria are of special interest since they function not only in the catching and paralysis of prey, but also in its digestion and ingestion (Collin, 1912; Kitching, 1952a; Hull, 1961a,b). In nature or in the laboratory, a sessile suctorian, e.g., *Tokophrya infusionum* or *Podophrya collini*, can easily catch vigorously swimming ciliates up to four times its own size. The ciliate must come in contact with the tip of only one of the numerous slender tentacles for it to be caught and soon immobilized. Whereas the adhesion phenomenon is elicited by every species of ciliate so far tested, it does not occur in response to any species of flagellate or ameba. Killed ciliates brought in contact with the tentacle tips did not elicit adhesion but Hull (1961a) has succeeded in producing adhesion with an "artificial ciliate" prepared by freezing and grinding a solution containing agar, acetylcholine, reduced glutathione, proteose-peptone, $CaCl_2$, and K_2HPO_4.

He has suggested that acetylcholine plays a fundamental role in the adhesion phenomenon, a hypothesis strengthened by the observation that pretreatment (before combining them) of either the prey ciliates or suctorians for 30 minutes with atropine or eserine inhibited the adherence phenomenon.

Electron microscopy of feeding *Tokophrya* has revealed a remarkable situation (Rudzinska, 1962b). The tentacle consists of two concentric tubes, an outer one bounded by the surface pellicle with its lumen continuous with the general cytoplasm and an inner one bound by a cylinder of seven bundles of seven fine tubules each and extending deep into the body of the organism. In the cytoplasm near the base of the tentacle and also in the outer tube occur small dense bodies of characteristic shape and structure (missile-like in sagittal section). A few of these have been seen protruding from the end of the nonfeeding tentacle, which in this region only is covered by the inner plasma membrane but not by the external pellicle. These bodies are more numerous in the outer tube in a feeding than in a nonfeeding tentacle. They almost surely correspond to the minute bodies seen by Hull (1961a) with dark field in living material streaming along the tentacle toward the prey. In electron micrographs of a feeding tentacle the inner tube is occupied by intact mitochondria and other cytoplasmic elements from the prey, evidently flowing in toward the *Tokophrya*. The missile-shaped bodies may well function in the attachment and paralysis of the prey and in the partial solution of its cytoplasm. The inward flow of food material is an active process requiring the expenditure of energy by the suctorian (Hull, 1961b).

The trichocysts of ciliates are much better-known aggressive structures (Grimstone, 1961). A discharged trichocyst consists of a long cross-striated shaft with a short spine at its tip. The unextruded trichocyst has no signs of shaft or cross-striation, yet it must contain within itself the inherent structure-building properties of fibrous proteins. Trichocysts have been assumed to develop in association with ciliary basal bodies (see Section III), but a recent electron microscope study of the regeneration of trichocysts in paramecia indicates that this is not so. Each trichocyst originates deep in the endoplasm from a homogeneous primordium within which the structures characteristic of the trichocyst gradually develop (Yusa, 1963).

Although the "trichocysts" of flagellates have been thought to be similar to those of ciliates, observations on *Chilomonas* with the electron microscope show that they are different. The peculiar and characteristic extrusible structures possessed by this flagellate have been named

ejectisomes by their discoverer (Anderson, 1962). It seems likely now that these structures, like the trichocysts of ciliates and like the missile-shaped bodies of *Tokophrya*, are not of surface origin, although of course their functions occur at the cell surface.

E. Locomotion

In protozoa locomotion is a function not only of the cell surface and structures associated with it, but also of special organelles arising from the cytoplasm near the cell surface.

The movement of amebae, akin to such cytoplasmic movement as cyclosis in plant cells, is of so much general significance in cell biology that it merits the separate treatment it has received in this series of volumes (see R. D. Allen, Chapter 3 in Volume II of this treatise). Movement of the ameboid type occurs to some extent in most groups of protozoa. It certainly depends on an energy source and probably on the presence of contractile proteins. It is worth noting that an ameba viewed from the side often may be seen to make contact with the substrate on which it is moving only at the ends of its pseudopods. It appears to "walk" along rather than glide.

Movement of a gliding type, without any apparent locomotory organelles, is prevalent especially among the sporozoa and has been best studied in the large gregarines, parasites of insects and other invertebrates. It semes likely that this movement is effected by the contraction of fine fibrillar aggregates visible by electron microscopy just below the surface of the organism. The deep folds of the surface pellicle of gregarines (see Section II, A) doubtless facilitate movement in much the same way as do the scales of snakes.

The cilia and flagella of protozoa, locomotory organelles providing a basis for classification of two great groups of protozoa, do not differ materially in fine structure from each other or from cilia and flagella found throughout the plant and animal kingdoms (cf. Fawcett, Chapter 4 in Volume II of this treatise; Fauré-Fremiet, 1961). The fundamental plan is a cylindrical structure formed by a continuation of the external membrane of the cell and containing within it a cylinder consisting of nine double fibrils about 150 A. in diameter. Along the axis of this central cylinder lie two additional fibrils of about the same diameter. Extending from one of each of the nine pairs of fibrils there may be two short arms, which make the whole structure asymmetrical. Other interesting modifications of the basic plan are being discovered as better preparations from a wider variety of organisms are studied.

Flagella in general are not only longer but considerably thicker

than cilia. The increased thickness results from the presence of additional structures. This is well shown in *Peranema*, each flagellum of which contains in addition to the bundle of eleven fibrils a relatively stout tapering rod (Roth, 1959). Furthermore, the interior-projecting one of the two flagella has a sheath of striated material located on the outside of the membrane in two crescent-shaped halves, and, external to this, a layer of fine low-density filaments, the mastigonemes. As a result, this flagellum has a diameter of 1 μ. Similar elaborations have long been known in flagella of other species, but not in cilia. A more general difference between cilia and flagella lies in the "wavelength" of the wave of bending, this being longer than the length of a cilium and shorter than the length of a flagellum (Child, 1961).

The movement of cilia and flagella, like ameboid movement, requires energy and is mediated by adenosine triphosphate (ATP). Recent lines of evidence supporting this statement are the following. Flagella prepared from a "paralyzed" mutant of *Chlamydomonas* had about one-third the adenosine triphosphatase activity of flagella similarly prepared from the normal strain (Brokaw, 1960). When the ciliates *Euplotes* or *Spirostomum* were partially cytolyzed with saponin or digitonin in an appropriate medium, preparations could be obtained which had coagulated endoplasm, were nonmotile, and did not recover on transfer to culture fluid. Yet if these partially cytolyzed structures were placed in the presence of ATP at 0.5×10^{-3} to 1.0×10^{-2} *M* together with Mg^{++} (1×10^{-3} gm. ion l) the cilia became motile (Seravin, 1961). In *Spirostomum* there was no coordination of the ciliary beat and consequently no translocation, but in *Euplotes* movement of the cirri produced walking of the "organism." When a preparation of cytolyzing *Spirostomum* was forcibly injected into a solution containing ATP and Mg^{++}, so that the organisms became disrupted, examination by dark field revealed isolated cilia performing rhythmic wavelike contractions; this activity lasted 10–15 minutes. In keeping with this demonstration of independent activity of each cilium is the finding of Lansing and Lamy (1961) that adenosine triphosphatase is localized next to two of the peripheral fibrils in the cilia of rotifers. Mackin (1958) has provided theoretical reasons why one must conclude that active contractile elements are present along the length of a flagellum; the movement cannot be regarded as that of a passive elastic element.

F. Coordination of Movement; Fibrillar Systems

Since protozoa may have several flagella and up to thousands of cilia, all moving in coordinated fashion, mechanisms must exist whereby

this coordination is effected. A physical basis for the coordination has long been sought, something akin to the nervous system of the metazoa; and in fact various fibrillar systems are visible by light microscopy, particularly in ciliates. In 1920 Taylor showed that cutting the larger rootlet fibrils in *Euplotes* brought about lack of coordination and interference with normal movement. Certainly there is a general correlation between the complexity of the rootlet system and the ability of the organism to perform a variety of movements. Thus *Euplotes* with a complex system can execute nine different kinds of swimming or creeping motions (Roth, 1958).

Although Jahn (1961) has shown that the phenomena of ciliary reversal and activation by electric current in *Paramecium* can be explained by assuming the ciliate to be a core conductor immersed in a volume conductor, without the need for assuming the existence of a neuromotor system, other phenomena are less readily explained. Whereas surgical removal of the entire macronucleus from a paramecium had no effect on coordinated ciliary movement, a transverse incision on the surface of the ciliate did disrupt coordination (Doroszewski, 1958). If two parallel transverse incisions were made, the cilia in this isolated field might move either more quickly or more slowly than those over the rest of the body. Interestingly enough, however, if reversal of motion occurred it included the isolated field, suggesting that different control mechanisms exist for timing and for direction of beat.

Where experimental observation (Parduez, 1958) and light microscopy indicated the existence of special structural systems for coordination of ciliary movement, electron microscopy is now in process of revealing structural details of such systems far more complex than anyone would have imagined. Each flagellum and each cilium has at its base, and probably is formed by, a basal body or kinetosome. The kinetosomes are cellular organelles of such general importance that they will require separate discussion (see below). Here it will suffice to say that the basal ends of the kinetosomes of ciliates are associated with some of the fibrillar systems which traverse the ectoplasm. In tetrahymenid ciliates, for example (Pitelka, 1961a), a tapering, striated fiber arises from the right anterior quadrant of each kinetosome at its base and passes anteriad and outward toward the pellicle. The fibers from adjacent kinetosomes in a ciliary row overlap to form the kinetodesmal fiber (Figs. 13 and 14). This lies to the right of each ciliary row, if one imagines that one is viewing the surface of the cell from inside the cell and always facing toward the surface. Also to the right of each ciliary row and just beneath the pellicle is

a flat longitudinal band consisting of fibrils or tubules about 20 mμ in diameter. Fibrils of the same appearance (postciliary fibrils) arise from the posterior right quadrant of each kinetosome, pass posteriad, and end near the next longitudinal fibril band to the ciliate's right. Extending left from each kinetosome is a transverse band of such fibrils

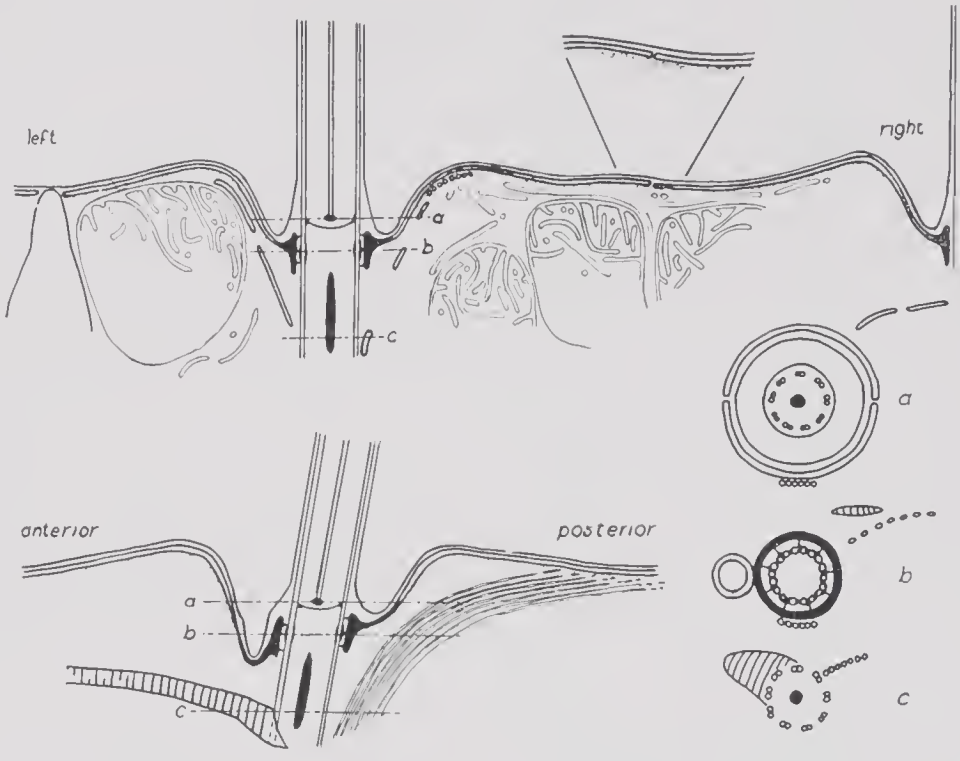


FIG. 13. Diagrammatic reconstructions of cortical structures in *Colpidium campylum*. Top: transverse section. Protrichocyst is shown in secondary meridian at left. Secondary meridian at right is enlarged above to show membrane configurations. To left of kinetosome are segments of two transverse fibrils; to right of kinetosome are cross sections of three kinetodesmal fibers. Fibrils of longitudinal band shown as circular profiles just under pellicle to right of kinetosome. Parts of several mitochondria and cytoplasmic membranes are included. Bottom left: idealized longitudinal section showing kinetodesmal fiber anterior to, and postciliary fibrils posterior to, kinetosome. Bottom right: cross sections of ciliary base at successively deeper levels as indicated by lines *a*, *b*, and *c* on other two diagrams. Transverse fibrils to left (below) cilia in sections *a* and *b*. Parasomal sac and fused pellicular ring shown in section *b*; kinetodesmal and posteiliary fibril attachments shown in section *c*. From Pitelka (1961a).

ending in the vicinity of the next longitudinal band to the left. Direct connections between these three sets of fibrillar bands have not been seen, whereas, as already noted, the longitudinal kinetodesmal band is formed by the overlapping of bundles of fibers arising from each of the kinetosomes.

The kinetodesmal fibers of *Stentor*, visible in the living organism,

are similarly formed from overlapping sheets of fibrils connected to the kinetosomes (Randall and Fitton-Jackson, 1958). If one follows a sheet anteriorly, individual fibrils may be seen to diverge from it and split into at least two subfibrils which get farther apart and are joined

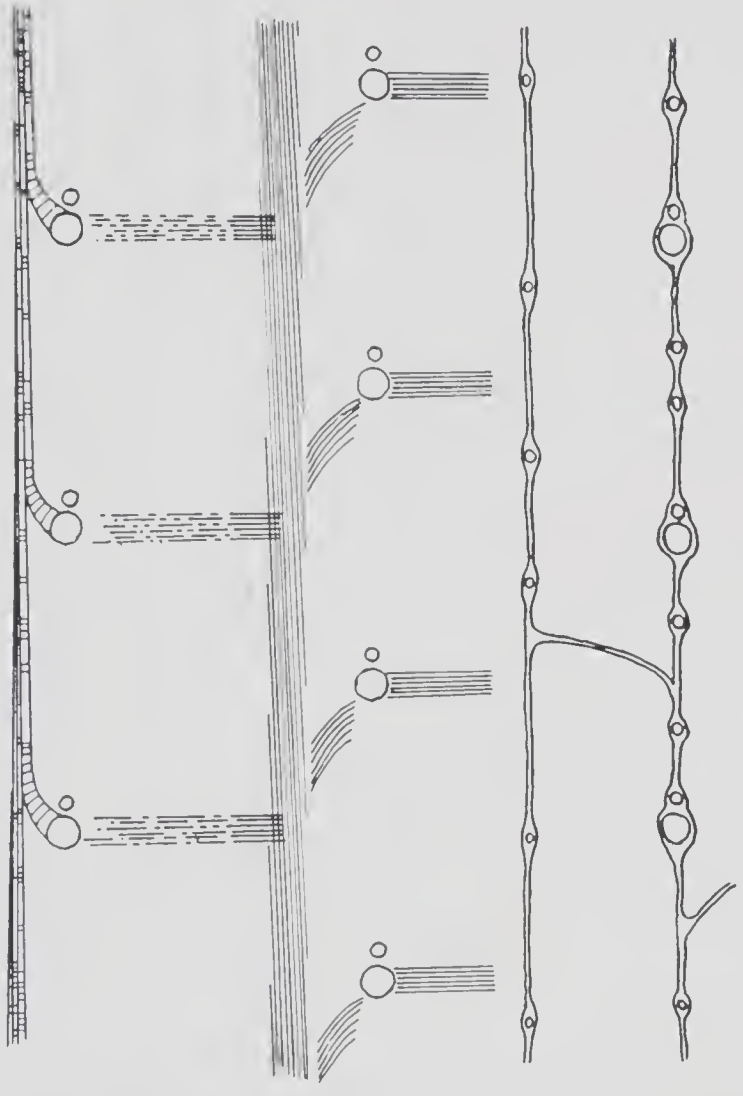


FIG. 14. Diagrammatic surface view of parts of three kineties of *Colpidium campylum*. Cilia indicated as empty circles, with parasomal sacs shown as smaller circles anterior to them. Kinety at left is shown with kinetodesmal fibers only. Kinety at center is shown with accompanying longitudinal, transverse, and post-ciliary fibrils. Kinety at right shows outlines of pellicular blisters along primary and adjacent secondary meridians; scattered small circles are protrichocyst attachments. From Pitelka (1961a).

by interconnecting filaments. These subfibrils curve toward the kinetosome and join it at opposite sides. Lying beneath and slightly to one side of each kinetodesmal fiber, and likewise visible in the living cell, are the so-called M bands, consisting of ill-defined filamentous material. Adjacent M bands are connected by side branches. The M bands

correspond to what have been called "myonemes" in the light microscopy of fixed and stained *Stentor*, whereas the kinetodesmal fibers are the "myonemes" seen with the light microscope in living *Stentor*. Whether these structures, or those of the tetrahymenids, function in contraction or conduction remains to be shown. The M bands of *Stentor* have an internal structure like that of smooth muscle and might represent a contractile cytoplasmic gel. The kinetodesmal fibers might then be the conducting and coordinating structures. It is possible that each kinetodesmal fiber is attached at each end to a different kinetosome, thereby providing a structural basis for ciliary coordination.

Further support for such a hypothesis is provided by the complex root fiber system of the adoral membranelles of *Stentor* (Randall and Fitton-Jackson, 1958). Each membranelle consists of three rows of 20–25 cilia per row. From the base of each membranelle of about 75 cilia, a fan-shaped bundle of root fibers converges to form a single cable. There are approximately 10 fibrils extending from each kinetosome, or 600–750 fibrils in all. The cables extend about 20 μ into the endoplasm and branch at their inner ends to form an interconnecting basal fiber. Again we see a perfect structural basis for coordination of the cilia in the membranelle.

The relationships of flagella and cilia to sensory organelles (Fauré-Fremiet, 1961) may find their origins among the protozoa. In *Euglena* one of the flagella is in close contact with the stigma (Wolken and Palade, 1953; Manton and Clarke, 1956). In the chrysomonad *Chromulina psammobia* one of the two flagella is very short, wholly intracellular, and in close contact with the stigma (Fauré-Fremiet, 1958). This structure is analogous to that in the rods of the vertebrate retina, where saccules containing the visual purple are in contact with sensory cilia.

III. Basal Bodies or Kinetosomes. Centrioles

Both flagella and cilia may be seen, by light microscopy of stained preparations, to arise from minute structures of solid appearance. These have generally been called basal bodies or blepharoplasts, or, in ciliates, kinetosomes. The fine structure of the basal bodies of flagellates and the kinetosomes of ciliates is identical, and rather different from what might have been expected. Each basal body or kinetosome is a short cylinder composed of a cylindrical arrangement of nine double or triple fibrils (Grimstone, 1961), which may continue distally as the nine double fibrils of the cilium or flagellum.

The basal body usually does not contain a central pair of fibrils, as does the cilium or flagellum, but it may contain a central rod with radiating spokelike structures connecting to the peripheral fibrils. The proximal or inner end of the basal body or kinetosome appears open to the cytoplasm, but the distal end ordinarily has a plate separating it from the base of the flagellum or cilium. One of the most interesting revelations of electron microscopy has been the demonstration that centrioles have a fine structure much like that of the basal bodies (Gall, 1961).

Centrioles are structures long seen by light microscopy in stained preparations of most kinds of animal cells but absent from cells of the

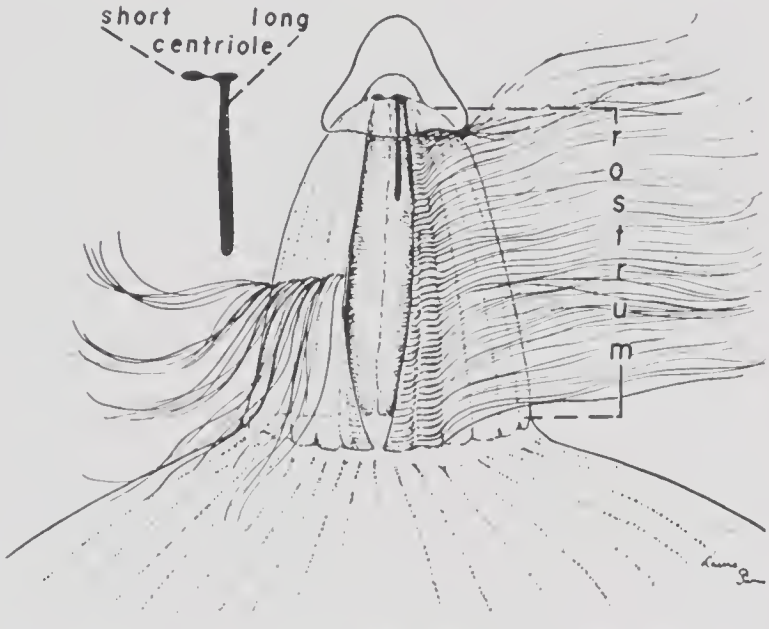


FIG. 15. Detail of anterior end of *Trichonympha* at very early stage in reproduction; rostrum opening up, but no activity of centriole (shown enlarged in inset). From Cleveland (1960a).

higher plants. They characteristically divide just before nuclear division and serve as centers for the spindle and astral rays during mitosis. In amebae there may be no centrioles or the centrioles may be intranuclear. In ciliates no centrioles have been seen associated with the micronucleus, but it is important to note that the kinetosomes are duplicated before cell division. Centrioles reach their greatest development among the flagellate protozoa. In the genus *Trichomonas*, for example, not only does the blepharoplast complex including the centriole serve for spindle formation and attachment of flagella, but it also gives rise to other organelles: axostyle, parabasal filament, and costa (Anderson and Beams, 1961). At cell division some of the organelles of the parent cell are carried over, but others are discarded and arise

anew from the centriole (Cleveland, 1961). The synthetic activities of the centriole are especially pronounced and have been observed in most detail among the hypermastigotes (Cleveland, 1960a). A non-dividing cell of *Trichonympha* has two centrioles, one short and one long (Fig. 15). At cell division the short one elongates, and each of the two centrioles now present forms at one end a new short centriole, and at its opposite end half the achromatic figure (Fig. 16). As the cytoplasm divides longitudinally the flagella and associated organelles of one side go to one daughter, those of the other side to the other daughter, while each new short centriole produces anew the flagella and associated organelles for the second side of each daughter cell. It

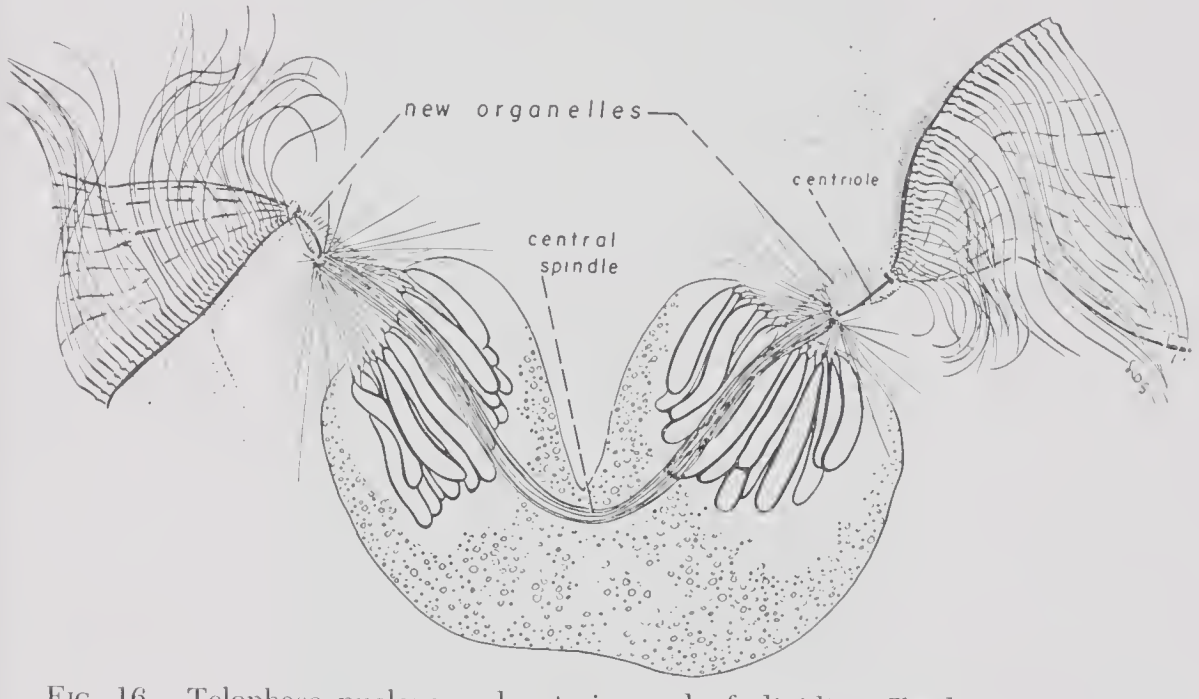


FIG. 16. Telophase nucleus and anterior end of dividing *Trichonympha*. From Cleveland (1960a).

is noteworthy that each of the numerous flagella so formed has its own basal body and that the new formation of all these basal bodies is initiated apparently through some synthetic activity of the centriole. In the gametogenesis of *Trichonympha* the activity of the centriole is even more pronounced, for here most of the organelles are lost and two complete sets are then regenerated by the centrioles (Cleveland, 1956a). The centrioles of *Trichonympha* can be seen in the living state (Fig. 17). In sections examined by electron microscopy the mature or long centriole appears to consist of a laminated half-disk with a slender rod extending into the rostral tube (Pitelka and Schooley, 1958). The second centriole is represented only by a half-disk.

The centrioles of *Trichonympha* are autonomous organelles (Cleve-

land, 1960a), as autonomous as the chromosomes and just as essential. Perhaps the most striking evidence in support of this statement has been provided by the observation of cell division without chromatin in the gametogenesis of *Trichonympha* (Cleveland, 1956b). In the presence

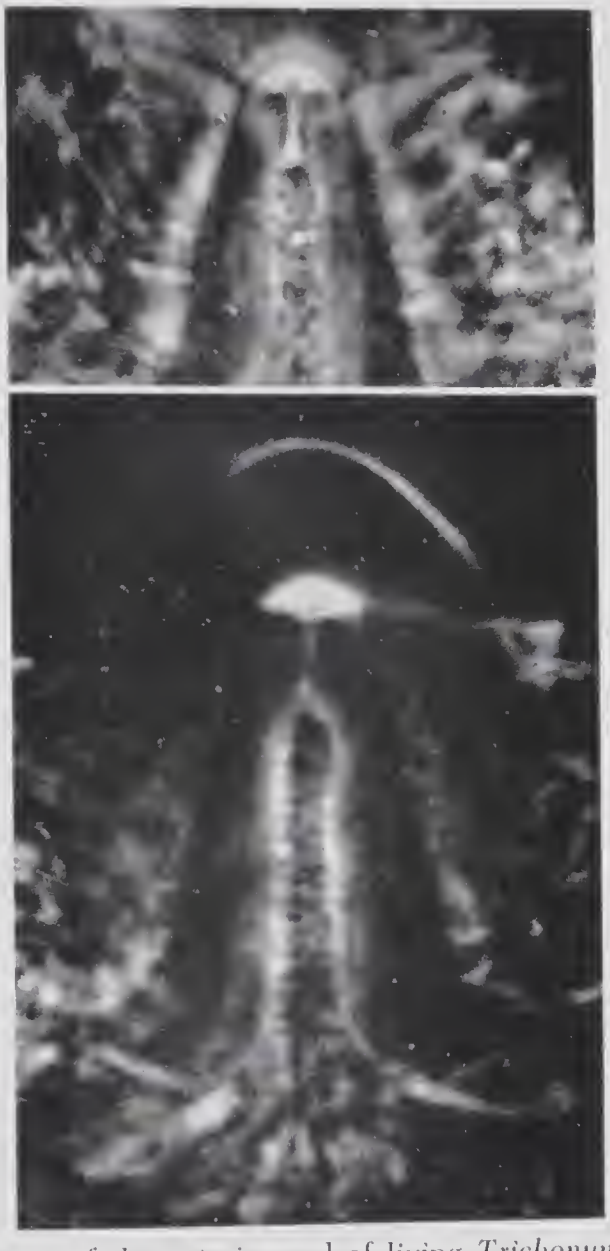


FIG. 17. Two views of the anterior end of living *Trichonympha collaris* to show the long and short centriole appearing bright beneath the bright cone-shaped inner cap; lower fig. shows the large, dark outer cap. Phase contrast. Magnification: × 250. From Cleveland (1960b).

of sublethal concentrations of oxygen the chromosomes were destroyed. The centrioles nevertheless produced the achromatic figure, flagella, and parabasal filaments, and the cytoplasm divided to produce two anucleate gametes.

How the centrioles accomplish all this, or how they replicate them-

selves, remains quite unknown. In the spermatocytes of the snail *Viviparus*, just before the pachytene stage, an immature or procentriole appears next to each of the two mature centrioles (Gall, 1961). Its structure resembles that of the mature centrioles (a cylindrical arrangement of nine triple fibrils) except that it is shorter, being annular rather than tubular. Its central axis is perpendicular to that of the mature centriole, which is assumed to have formed it. Each mature centriole is a polarized structure, one end forming the flagellum or other centriole products, the other end presumably being concerned with replication. This is a situation much like that already described for the centrioles of *Trichonympha*.

In flagellates having numerous flagella (as *Trichonympha*) the basal bodies underlying these flagella apparently are derived from the centriole, and there are stages in the life cycle when only the centriole persists, the basal bodies as well as their flagella having been resorbed. In ciliates, however, which lack centrioles, the basal bodies or kinetosomes persist even when the cilia are lost, and at cell division they appear to be duplicated directly from pre-existing kinetosomes. The kinetosomes of ciliates behave during morphogenesis in a manner reminiscent of the cells of metazoa during embryonic development or regeneration (Tartar, 1961). The kinetosomes increase in number and become arranged in characteristic patterns or fields. Lwoff (1950) has attributed to them not only autonomy, but also the main morphogenetic role in formation of all the ectoplasmic organelles. We have already noted (Section II) that the trichocysts, considered by Lwoff to be formed, like cilia, from kinetosomes, in fact arise from special primordial vesicles deep in the endoplasm. There is at present no basis on which to attribute to kinetosomes a role in anything more than the formation of more kinetosomes, of cilia, and of the associated fibrillar systems, just as centrioles have a role in forming more centrioles, spindle fibers and astral rays, axostyles, parabasal filaments and other fibrillar elements, flagella, and basal bodies which in turn can form flagella. These are roles enough!

Observations by electron microscopy on the development of basal bodies in regenerating or growing *Stentor* show clearly that nothing resembling the conventional conception of division of these bodies occurs (Randall and Hopkins, 1962). In the earliest recognizable stage the basal bodies are ringlike structures with a core less dense than the shell. This appearance is followed by an arrangement of small vesicles and then by fibrogenesis. The existing basal body perhaps produces substances which under the influence of materials and conditions in the adjacent cytoplasm assume the forms and functions characteristic of the basal body.

A concept of molecular morphogens is fundamental to an ultimate understanding of differentiation. For the experimental study of such a concept the fibrous proteins provide an exceptionally good approach; the morphogenesis of such proteins from the kinetosomes and centrioles of ciliates and flagellates may well be the material of choice for this approach (Berrill, 1961). First much more will have to be discovered concerning the chemical nature of kinetosomes and centrioles as well as of the fibrils they seem to form. Although both DNA and RNA are said to be present, they have been noted only at the limits of detectability, and there is much room for error. Kinetosomes prepared by a centrifugal fractionation method (Seaman, 1960) had a variety of enzymatic activities and appeared to synthesize protein (Seaman and Gottleib, 1957). The purity of these preparations, however, is open to some question. Much more work of this kind needs to be done.

IV. Mitochondria

The chondriome of protozoa varies from long, branching structures, seen especially in some flagellates, to more or less numerous short, rod-shaped or nearly spherical bodies, as seen in many ciliates (Grassé, 1953). Time lapse photography has repeatedly and beautifully demonstrated the extreme plasticity of the chondriome; mitochondria, far from being rigid structures, are flexible, constantly changing in shape and position as befits organelles of their intense biochemical activity. This activity is reflected in their specific staining reactions and in the enzymatic reactions they catalyze, which constitute the cytophorase system. The relatively small amount of biochemical work so far done with mitochondria isolated from protozoa serves only to indicate that in general they resemble mitochondria from other organisms (Klein and Neff, 1960).

Also in their fine structure they resemble mitochondria from other sources (Grell, 1962). Typically they are bounded by two membranes, a relatively smooth outer one and an inner one having numerous protrusions into the interior of the organelle. In flagellates these protrusions take the form of cristae (Fig. 18), just as in most vertebrate cells, but in most ciliates and amebae they are finger-shaped villi (Sedar and Rudzinska, 1956, and many subsequent studies) (Fig. 19). In the giant ameba *Pelomyxa* one end of each mitochondrion contains stromal material with only a few villi, but in the remainder of the mitochondrion there is a complex pattern of wavy intertwined villi, sometimes branching and fusing with each other (Pappas and Brandt, 1959). In the intraerythrocytic forms of malaria parasites, mitochondria are relatively

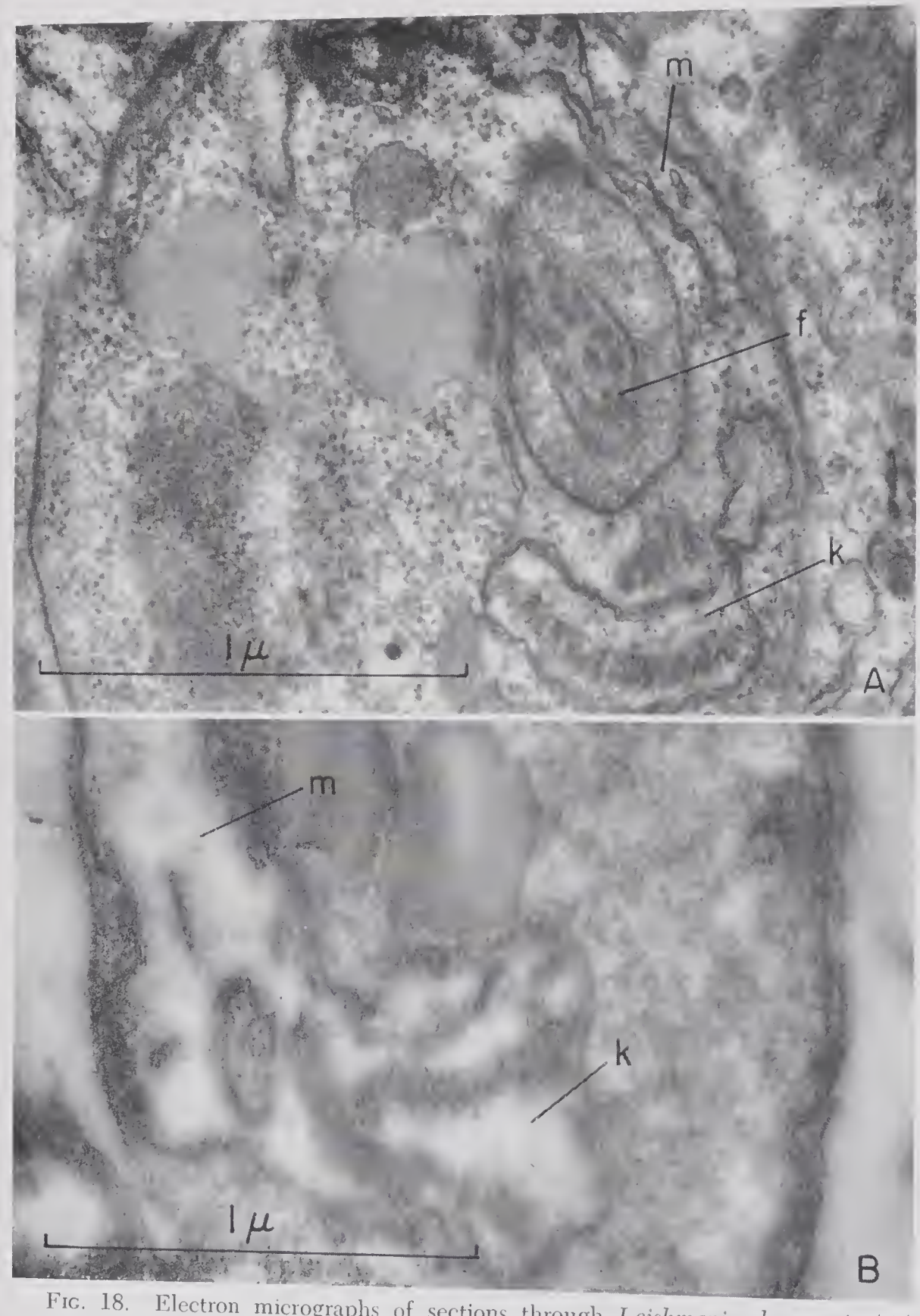


FIG. 18. Electron micrographs of sections through *Leishmania donovani*. (A) Leishmanial form in spleen. (B) Leptomonad formed after 19 hours *in vitro*. *K*, kinetoplast; *m*, mitochondrion; *f*, cross section of flagellum. Note in (B) continuity of kinetoplast and mitochondrion. From Rudzinska, D'Alesandro, and Trager, unpublished.

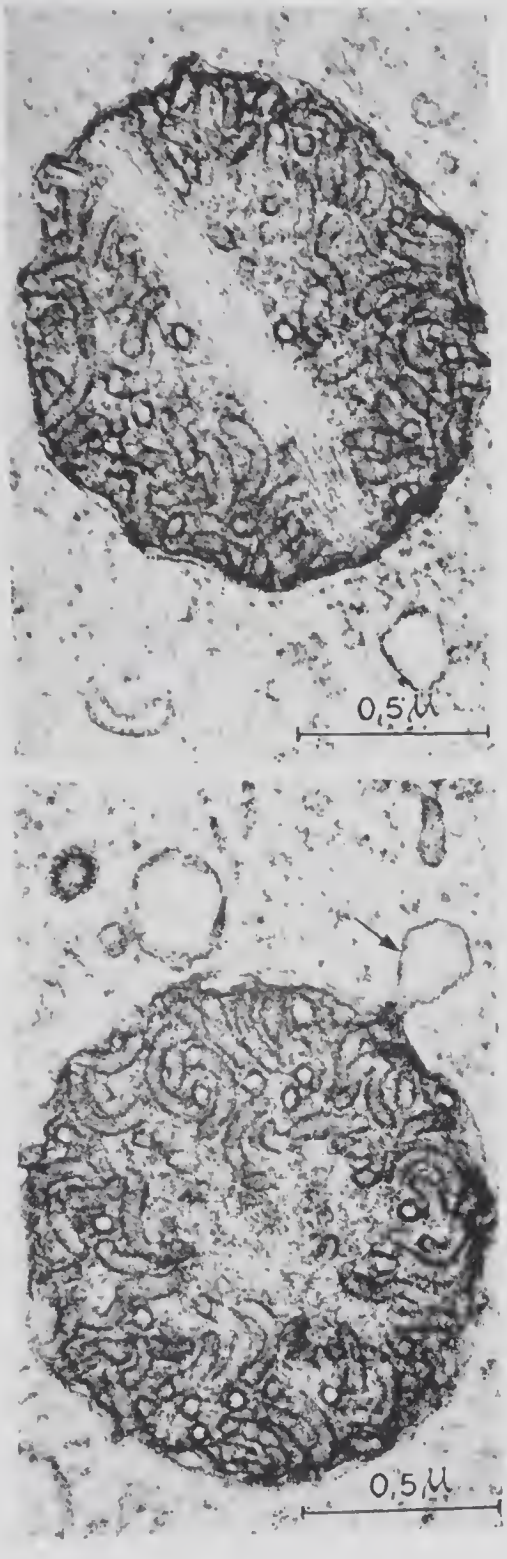
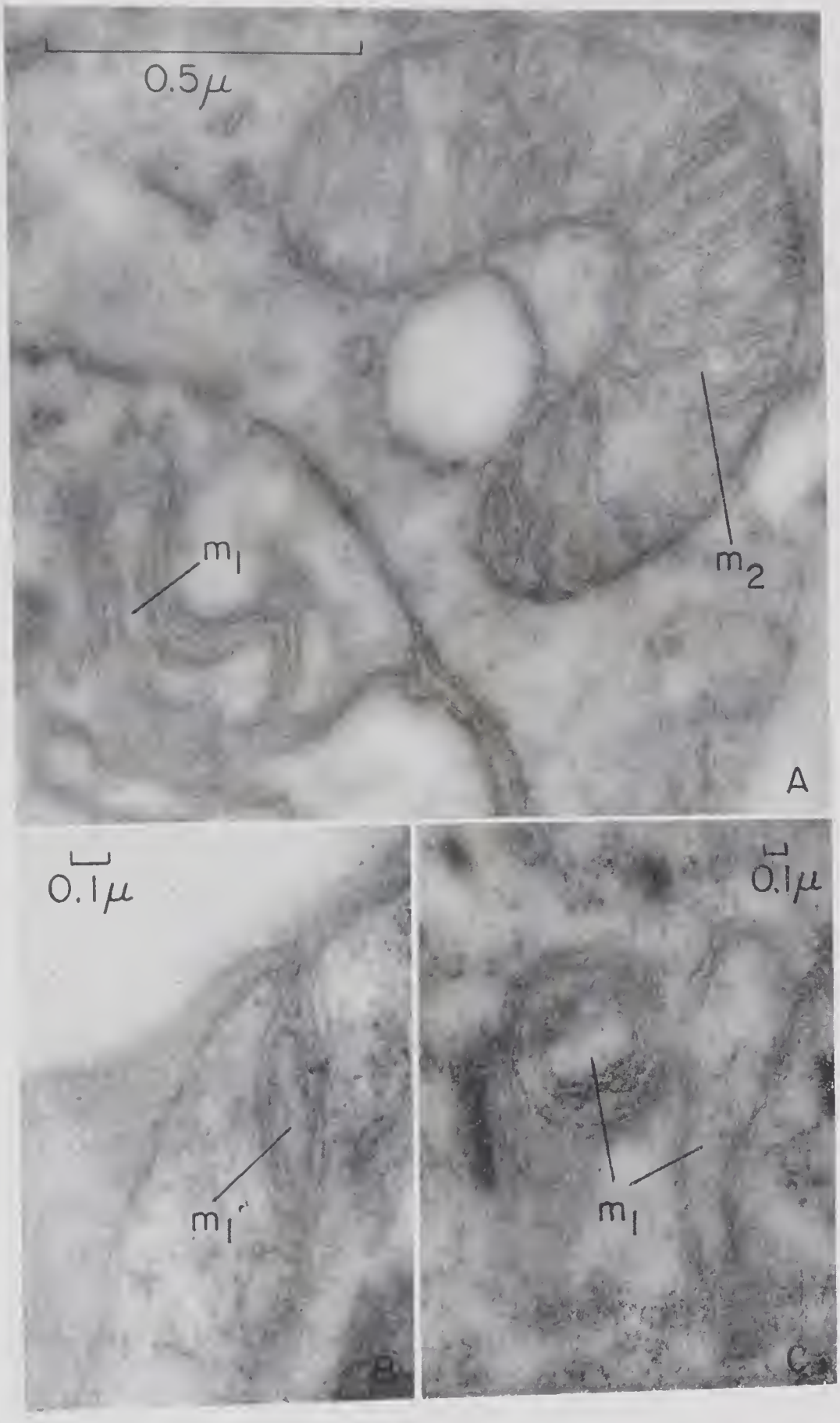


FIG. 19. Mitochondria of *Epistylis*. Electron micrograph of thin section. Upper figure shows material consisting of parallel fibers. Lower figure shows an outward protrusion of the external membrane (arrow). From Fauré-Fremiet *et al.* (1962).

few and their form varies with the species. In the avian parasite *Plasmodium lophurae* and in the human parasite *P. falciparum* each mitochondrion has a comparatively small number of villi protruding into its lumen, whereas in the rodent parasite *P. berghei*, the human parasite *P. vivax*, and the simian parasite *P. knowlesi* no typical mitochondria are present (Rudzinska and Trager, unpublished). Instead we see in each organism a few concentric double-membraned structures (Fig. 20) resembling mitochondria found in some stages of spermatogenesis in *Helix* (Beams and Tahmisian, 1954) and a double-membraned organelle of the giant bacteria *Thiovulum* (Fauré-Fremiet and Rouiller, 1958). Like the latter, the structures in *P. berghei* may be seen connected with the double plasma membrane of the cell, from which they perhaps arise (Fig. 20).

In the symbiotic intestinal flagellates of termites and the intestinal amebae of the genus *Entamoeba* no mitochondria have been found by electron microscopy (Grimstone, 1961; Miller *et al.*, 1961; Deutch and Zaman, 1959). This is in keeping with the obligate anaerobic mode of life of these protozoa. Less easily accounted for is the reported absence of mitochondria from the bloodstream-inhabiting parasite *Trypanosoma equiperdum* (Anderson *et al.*, 1956). The bloodstream forms of some trypanosomes do, however, have a peculiar and characteristic metabolism to which we will refer again in Section V.

The mitochondria of protozoa, like those of other cells, are sensitive to the physical conditions to which they are exposed as well as to altered physiological and pathological states of the cell (see Novikoff, Chapter 5 in Volume II of this treatise; Rouiller, 1960). In irradiated protozoa the mitochondria are smaller, just as they are in the liver of starved rats. The mitochondria of *Tetrahymena* have been observed to change with the phase of growth of the culture (Bak and Elliott, 1962). In the phase of logarithmic increase the mitochondria were elongate and near the surface. By the fifth day of the culture, when the stationary phase began, the mitochondria became rounder and were deeper in the cytoplasm. During the next 5 days electron-dense bodies appeared in many of the mitochondria and enlarged to fill some of them completely. A somewhat similar change in fine structure of mitochondria has been observed at encystation in *Acanthamoeba* (Vickerman, 1960, 1962a). The mitochondria of the trophic amebae have tubular cristae with often a single small spherical inclusion inside a crista. The inclusions have a diameter of 500–1000 A. and are either completely electron dense or with a dense cortex. In cysts these intracristal inclusions are much larger (0.3–0.5 μ diameter), and more opaque than the matrix and contain at their periphery electron-dense granules 50–300 A. in size and of irregular shape. It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A
0.1μ
m1'
B
0.1μ
m1
C

would be of much interest to compare the biochemical properties of mitochondria prepared from the cysts and from the trophic amebae. For studies of such a nature, relating biochemical properties to ultrastructure and to metabolic activity of the intact cell, protozoa provide exceptionally favorable material.

One might also expect protozoa to be particularly useful in attempts to determine the mode of origin of mitochondria. Many years ago Fauré-Fremiet (1910) described the simultaneous division of the mitochondria and the micronucleus in the ciliate *Urostyla*. Rouiller (1960) considers that the multiplication of mitochondria by division has been firmly established both by microcinematography and by electron microscopy. In algae of the genus *Micromonas* the single mitochondrion divides during cell division (Manton and Parke, 1960), and Ris (1962) has detected in this mitochondrion 25 A. filaments which might be deoxyribonucleic acid (DNA). Other workers, however, consider the origin of mitochondria in protozoa as well as in other cells to be obscure, and they doubt whether they are typically autonomous self-replicating (or better self-dependent (Ris, 1962)) organelles (Grimstone, 1961).

Among several other hypotheses, it has been suggested that mitochondria originate from the cell membrane by a process of infolding and pinching off. There is good evidence for this in the malaria parasite *Plasmodium berghei* (see above), but the structures so formed are far from typical mitochondria. Still another hypothesis assumes origin of the mitochondria from small undifferentiated vesicles in the endoplasm, and an arbitrarily arranged series of stages illustrating such development in *Paramecium* has been figured by Wohlfarth-Botterman (1957). Perhaps of more general significance is the hypothesis of nuclear origin. This has received support in electron microscope studies; in a variety of actively dividing tissues (rat thymus, mouse lymph node and spermatocytes, onion root tip) the mitochondria appear to form from folds of the nuclear membrane (Hoffman and Grigg, 1958). Similar but more convincing pictures have been presented by Brandt and Pappas (1959) in an electron microscope study of the ameba *Pelomyxa*. Regions of continuity can be seen between the double nuclear membrane and the double membrane of mitochondria. Either the mitochondria are fusing with the nucleus or they are originating from it.

FIG. 20. Sections through *Plasmodium berghei* in rat reticulocyte. (A) Edge of parasite showing parasite "mitochondrion" (m_1) and mitochondrion of reticulocyte (m_2). (B) Formation of parasite "mitochondrion" from the double plasma membrane. (C) Cross and longitudinal sections of parasite "mitochondria." Electron micrographs. From Rudzinska and Trager (1959).

V. The Kinetoplast

The apparent formation of mitochondria in certain flagellates from a special structure, the kinetoplast (or kinetonucleus) is of so much potential significance not only with respect to the origin of mitochondria, but also in relation to the whole problem of self-dependent cellular organelles, as to merit detailed discussion. Moreover, the kinetoplast (incorrectly termed "parabasal body" by some authors) is an organelle restricted in occurrence to certain nonpigmented flagellate protozoa. Such specialized organelles often provide valuable clues to generalized cellular functions—they represent a naturally occurring sequestration of function that would be difficult to achieve experimentally.

In free-living flagellates of the family Bodonidae and in the parasitic flagellates of the Trypanosomatidae there is usually present near the base of the flagellum a small oval structure which stains with the usual nuclear stains, is Feulgen positive (Bresslau and Scremin, 1924; Robertson, 1927), and contains DNA (Cosgrove and Anderson, 1954). This structure, the kinetoplast, lies adjacent to but is entirely distinct from the basal body of the flagellum (Fig. 18). At cell division, the kinetoplast ordinarily divides before the nucleus and at the same time that replication of the basal body and flagellum occurs. However, the synthesis of DNA in the kinetoplast takes place simultaneously with the synthesis of DNA in the nucleus, at least in the culture form of *Trypanosoma mega*, the one species in which this has been studied (Steinert and Steinert, 1962). This has been taken as evidence favoring the binucleate theory of Hartmann (1907). It is of interest in this connection that studies of a similar nature with labeled thymidine have shown that in two species of ciliates DNA synthesis in the macronucleus and micronucleus occurs at markedly different times—and this despite the fact that the macronucleus develops from a micronucleus and is genetically related to the micronucleus (Prescott *et al.*, 1962; McDonald, 1962). The kinetoplast, on the contrary, is never derived from the nucleus of the cell (Hoare, 1954). The simultaneous synthesis of DNA by it and the nucleus may be analogous to the simultaneous synthesis observed in the two macronuclei of fused doublets of *Euplotes* (Kimball and Prescott, 1962) and may therefore be determined by the cytoplasm.

Early observers of the kinetoplast noted that it reacted with mitochondrial as well as with nuclear stains (Shipley, 1916). But it remained for electron microscopy to show that in several species of Trypanosomatidae (Clark and Wallace, 1960; Steinert, 1960) and in *Bodo* (Pitelka, 1961b) the kinetoplast consists of two parts of different structure but en-

closed within the same membrane (see Fig. 18). One part appears as a comblike array of electron-dense fibrillar material; this is assumed to be the DNA-containing material, though this has not yet been proved. Projecting and seeming to grow from the clear membrane-enclosed space within which this dense structure lies are typical mitochondrial structures with typical cristae. That the kinetoplast gives rise to mitochondria therefore seems a reasonable hypothesis (Schulz and MacClure, 1961). The kinetoplast may represent what was originally a part of the nucleus that has become separated and specialized for this function and perhaps for others not yet guessed at.

In the genus *Trypanosoma*, most of which spend part of their life cycle in the blood of a vertebrate host and part in an invertebrate vector, only one species without a kinetoplast exists in nature—*T. equinum* (Hoare, 1954; Mühlpfordt, 1959). There are known two additional species, *T. equiperdum* and *T. evansi*, which readily give rise spontaneously to akinetoplastic strains. These three species are the only trypanosomes that do not have an intermediate invertebrate host; they are transmitted venereally, or by flies acting as "flying pins" (without cyclical development). In several other species of trypanosomes, treatment with sublethal doses of appropriate drugs, such as acriflavine, produces partially or completely akinetoplastic strains; these can be maintained in experimental animals by blood passage (Mühlpfordt, 1959). Akinetoplastic trypanosomes are however unable to infect the intermediate host (Reichenow, 1940). The bloodstream forms of trypanosomes have not been obtained in culture (Von Brand, 1952), and it has not been possible as yet to produce viable akinetoplastic strains of the culture forms that correspond to forms found naturally in the invertebrate vector. Culture forms and bloodstream forms of the African trypanosomes differ sharply in their metabolism (Von Brand *et al.*, 1955; Ryley, 1962); the respiration of the bloodstream forms is not sensitive to cyanide. It will be apparent that all these facts fit the hypothesis that the kinetoplast forms the mitochondria of these organisms. When the kinetoplast is lost only those forms can persist which do not have a cyanide-sensitive metabolism and hence have no need for functional mitochondria. Furthermore these forms can live and multiply only in the special environment provided by the vertebrate blood stream, an environment not yet duplicated *in vitro*. Reichenow (1940) had already suggested over twenty years ago such a metabolic function for the kinetoplast—that it could be dispensed with under the nutritive conditions of the blood but was essential in the alimentary tract of the vector. It is of interest that electron micrographs of sections of blood-

stream forms of the African trypanosome *T. gambiense* show the kinetoplast connected to a long canal which does not have a mitochondrial type of structure (Mühlpfordt and Bayer, 1961). Similarly, the bloodstream form of *T. rhodesiense* has a much less developed mitochondrial system than the culture form of the same species (Vickerman, 1962b). These facts, and the apparent absence of mitochondria in *T. equiperdum* (see above), are again in keeping with the hypothesis.

Recent work with another type of trypanosomatid, *Leishmania donovani*, provides still further support. This flagellate, the agent of human kala azar, lives in its vertebrate host as an intracellular ovoid so-called leishmania form, but in the alimentary tract of its sand fly vector it grows as a typical flagellate form, the leptomonad. The intracellular leishmanias, like the bloodstream forms of the African trypanosomes, have not yet been grown *in vitro* at 37°C., whereas the leptomonads grow readily at 28°C. in complex media containing blood (see Trager, 1953). If the leishmanial stages are removed from the spleen of an infected animal and held at 28°C., they change into leptomonads within about 12 hours. By electron microscopy it has now been found that whereas the leishmanial form has a kinetoplast in which the dense fibrillar structure is the predominant element and has few mitochondria, the metamorphosing forms present after only 5 hours at 28°C. have kinetoplasts with very large mitochondrial elements protruding from them (Rudzinska *et al.*, 1962) (Fig. 18). Such a kinetoplast and numerous large branching mitochondria also characterize the leptomonads. Again it would appear as if some metabolic function of the kinetoplast and mitochondria formed from it, inhibited at 37°C., is compensated for by the intracellular mode of life.

A somewhat analogous but simpler situation has been reported in the yeast *Torulopsis* (Linnane *et al.*, 1962). When grown aerobically it has typical mitochondria with numerous cristae; these mitochondria when isolated showed all the biochemical activities characteristic of mitochondria. Under anaerobic conditions mitochondria are absent, but there are membrane systems connected to the nucleus. These can be seen giving rise to mitochondria in yeasts transferred from an anaerobic to an aerobic environment.

Although the hemoflagellates are a more difficult material to handle than yeast, they provide the special advantage, already noted earlier, that a specialized organelle, rather than the whole nucleus with its numerous diverse functions, is concerned in mitochondrial formation. The kinetoplast of the hemoflagellates should repay detailed study as a material for the investigation of such fundamental problems in cell biology as

the mode of reproduction of DNA-containing self-dependent organelles and the origin of mitochondria.

VI. Chloroplasts

The phytoflagellates resemble algae in having chloroplasts with chlorophyll and other pigments and in carrying on photosynthesis; they resemble flagellate protozoa in other respects. A variety of colorless flagellates exist in nature which correspond closely to different kinds of phytoflagellates except for their lack of pigment. Since, furthermore, permanently or temporarily bleached strains of phytoflagellates can be produced experimentally, these organisms are excellent for the study of chloroplast formation, reproduction, and function.

The properties of chloroplasts have already been discussed in detail in Volume II of this treatise (see Granick, Chapter 7). For the chloroplasts of phytoflagellates there is general agreement that ordinarily they arise by division from preexisting chloroplasts. In *Chlamydomonas* the relatively large chloroplast has one or more irregularly shaped bodies, usually near the pyrenoid, which contain DNA, as shown by Feulgen and acridine orange staining and by sensitivity to deoxyribonuclease (Ris and Plaut, 1962). In electron micrographs these bodies show fine fibrils 25 A. in thickness, as well as much granular material.

Cytoplasmic DNA probably associated with the chloroplasts has also been demonstrated in *Euglena gracilis* (Sagan and Scher, 1961; Scher and Sagan, 1962). Dark-grown as well as light-grown euglenas incorporated labeled thymidine into the cytoplasm, whereas euglenas bleached with streptomycin or ultraviolet did not. Presumably in the dark-grown euglenas proplastids were nevertheless actively growing and producing new DNA. The growth and enzymatic activities of proplastids in euglenas have recently been studied in detail by Gibor and Granick (1962). The proplastids of different "bleached" strains had varying degrees of ability to synthesize porphyrins, roughly proportional to the carotenoid content of the strain. Thus the plastids of two strains with the least carotenoids did not form porphyrins even when the cells were incubated with δ-aminolevulinic acid, whereas two strains of intermediate carotenoid content formed porphyrins only in the presence of this compound, and a strain of high carotenoid content, like normal green strains, formed porphyrins in the absence of added δ-aminolevulinic acid.

The existence of proplastids and their multiplication in the cell under conditions not permitting synthesis of chlorophyll does not fit well the hypothesis of the origin of chloroplasts from symbiotic chlorophyll-containing microorganisms. Blue-green algae in the dark are not known

to shrink to the state of a proplastid. It seems more likely that the DNA of chloroplasts, like that of the kinetoplast of hemoflagellates, represents a specialized portion of the nuclear DNA which, in the course of evolution, became independent of the nuclear DNA insofar as its formation is concerned. Comparative studies of the DNA of chloroplasts and the nuclei of the cells containing them, and of kinetoplasts and the nuclei of their cells, would reveal relationships of fundamental interest.

VII. Symbiotes in Protozoa

Whether chloroplasts or kinetoplasts or the "*Nebenkern*" of certain amebae originated as symbiotic intracellular microorganisms are moot questions. But there is no doubt as to the existence today of intracellular microorganisms specifically associated, and always occurring, with particular species of host protozoa. As with similar intracellular organisms associated with other kinds of invertebrates, there is good reason to believe that these symbiotes live in a state of mutualism with their hosts, each deriving benefit from the other (see Trager, Chapter 4 in Volume IV of this treatise). The symbiotes of protozoa are algae, bacteria, or bacteria-like structures. Among the algae the zoochlorellae of *Paramecium bursaria* have been most studied (see Chapter 4 in Volume IV). Less well known, but potentially even more interesting, is the association between a flagellate (*Cyanophora paradoxa*) and a blue-green alga, an example of a class of such associations called "syncyanoses" (Geitler, 1959). Each flagellate ordinarily contains 2 to 4 algal cells having the typical structure of blue-green algae (Figs. 21 and 22). As with all such associations, a controlling mechanism must be present to synchronize cell division of host and symbiote so that neither outgrows the other. Neither the blue-green alga nor the flagellate have yet been cultured apart from each other, an important first step toward studying their nutritional relationships.

More common than associations with algae are specific associations of protozoa with bacteria (Kirby, 1941). The bacteria may be on the surface, as is true of the so-called rods of *Lophomonas striata* (Beams *et al.*, 1960) or they may be internal (Roth and Daniels, 1961). Except for the extensive work with kappa and related particles in paramecia (see Chapter 4 in Volume IV; and Sonneborn, 1959) these associations have been little studied experimentally. In the ciliates *Euplotes patella* and *E. eurystomus* all individuals of all strains contained chains of fine Feulgen-positive granules (Fauré-Fremiet, 1952). These disappeared within 3–4 days after exposure of the ciliates to penicillin (50 units or

more per milliliter). Such treated ciliates divided once, rarely twice, and then cytolyzed 10–12 days after treatment, suggesting an essential role of the presumed bacteria, perhaps of nutritional nature.

Such a role is now well documented for bacteria-like structures occurring within the flagellate *Crithidia* (*Strigomonas*) *oncopelti*, an inhabitant of the alimentary tract of certain species of plant bugs. Newton (1957) noted that these flagellates could be grown axenically in a relatively simple medium containing only a few amino acids and two or three vitamins, whereas all other protozoa so far grown in defined media have much more complex requirements (as for example the closely related *Crithidia culicidarum* (Cowperthwaite *et al.*, 1953) and *Leishmania tarentolae* (Trager, 1957). Newton and Horne (1957) also noted the presence in *C. oncopelti* of so-called bipolar bodies containing RNA. Gill and Vogel (1962) then showed, in an ingenious experiment, that this flagellate synthesized lysine via α,ε-diaminopimelic acid in the manner characteristic of bacteria, and that furthermore this synthesis was associated with the "bipolar bodies." Proof that these bodies are in fact endosymbiotic bacteria has now been provided by Gill and Vogel (1963). In electron micrographs of thin sections these bodies show structures typical of bacteria, including stages in binary fission. The flagellates could be "cured" of their symbiotes by growing them in a peptone-liver medium containing penicillin. It will be of great interest to compare the nutritional requirements of such symbiote-free flagellates with those of the naturally occurring symbiote-containing ones. One may expect a situation parallel to that already demonstrated in several different kinds of insects (see Chapter 4 in Volume IV). Of equal interest would be the separate cultivation of the symbiotic bacteria and a study of their nutritional requirements. Their presumably great synthetic abilities are difficult to reconcile with an obligate intracellular mode of life, unless perhaps, like malaria parasites and rickettsiae, they require external sources of certain coenzymes (see Chapter 4 in Volume IV; and Moulder, 1962).

VIII. Cytoplasmic Inclusions

Among the many kinds of protozoa, a wide variety of inclusions occurs; they range from stored food to waste products, from amorphous bodies to crystals (Thomas, 1961), and about most of them very little is known. The particulate reserve polysaccharides of protozoa may be starch, consisting either of a mixture of amylose and amylopectin, as in the flagellate *Polytomella* (Barker and Bourne, 1955), or of amylopectin

FIG. 21. Section through *Cyanophora paradoxa* showing two of the blue-green algal cells within the flagellate. Electron micrograph. Magnification: × 33,000. From Hall (1963).

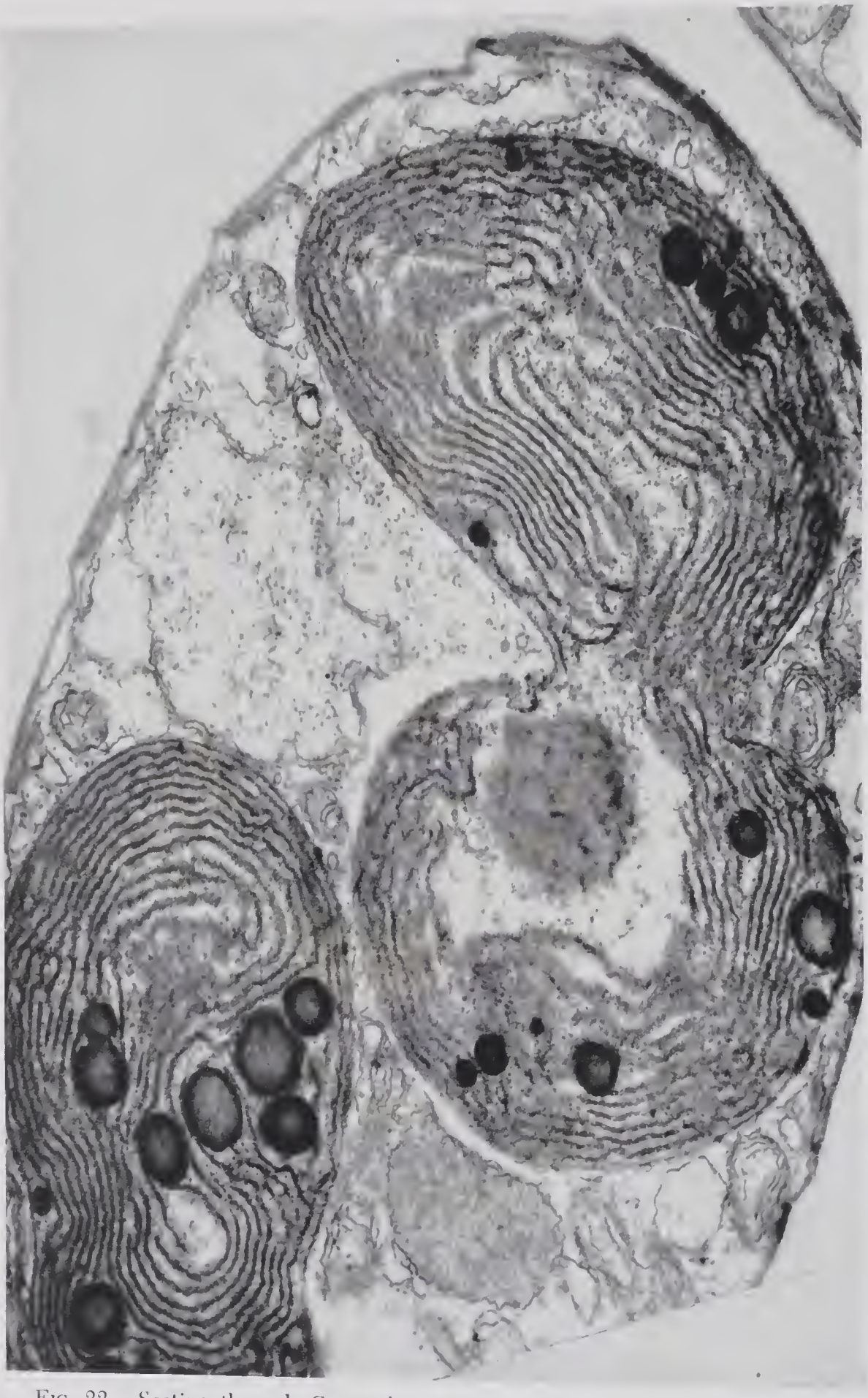

FIG. 22. Section through *Cyanophora paradoxa* showing algal cells in division. Electron micrograph. Magnification: × 33,000. From W. T. Hall, unpublished.

alone as in certain ciliates found in the intestine of horses and the rumen of sheep (Forsyth and Hirst, 1953), or they may be glycogen, as in *Tetrahymena* (Ryley, 1952) and many other species. The chemical nature of the volutin granules (Hall, 1953) found in many protozoa has not been well defined, nor is much known as to their function. They vary in number and size with the physiological state of the cell and are distinguished by their staining reactions. In trypanosomes (Ormerod, 1961) the culture forms are usually loaded with such granules, whereas bloodstream forms, though growing just as actively, are free of such inclusions or have relatively few (Ryley, 1962). This is no doubt an additional reflection of the fundamental differences in metabolism between these two forms. Among the strains of *Trypanosoma rhodesiense* there seems to be a positive correlation between virulence and lack of volutin granules (Ormerod, 1960) suggesting an approach to a study of virulence in relation to the metabolism of the parasite.

Another type of cellular inclusion is the chromatoid body in the cysts of parasitic amebae. The chromatoid bodies of *Entamoeba invadens* have a characteristic honeycomb-like ultrastructure (Deutsch and Zaman, 1959). They consist of ribonucleoprotein and arise by a process of aggregation of groups of 250–300 A. units to form polycrystalline masses in precystic and early cystic stages (Barker, 1963). These small precursor units are present in axenically grown *E. invadens* (which never encyst), and are composed of a variable number of lamellae 250–300 A. wide arranged in parallel arrays. Each lamella consists of coiled fibrils forming a closely packed helix (Siddiqui and Rudzinska, 1963). This is the first demonstration by electron microscopy of helical structure in a ribonucleoprotein particle.

Some protozoa produce characteristic pigments (other than the chlorophylls and carotenoids of the phytoflagellates). *Stentor coeruleus*, a large free-living ciliate appearing green by transmitted and red by reflected light, contains two pigments, one red-fluorescent and extractable with alcohol, the other having neither of these properties (Moeller, 1962). Both pigments belong to the *meso*-naphthadianthrone group and have been prepared pure in milligram amounts. Also belonging to the same group is the zoopurpurin of another large free-living ciliate, *Blepharisma*. Ciliates of this genus have a beautiful pink to purple color. The fluorescent pigments of *Stentor* and *Blepharisma* are phototoxins against other ciliates, including the stentors which produce it (Giese, 1954; Moeller, 1962). Whether this property is related to the function of the pigment in nature is doubtful. Perhaps the pigments play a role in orientation to a gradient of light; stentors swim away from light.

IX. Endoplasmic Reticulum; Golgi, Parabasal Bodies

In the preceding sections we have discussed the surface of the protozoan cell and some of the many discrete structures lying in the cytoplasm. It must not be thought that this cytoplasm is itself a structureless, inert matrix. When viewed by electron microscopy, the cytoplasm has an intricate structure consisting of complex systems of membrane-lined spaces and tubules—the endoplasmic reticulum (see Porter, Chapter 9 in Volume II of this treatise). Although there can be little doubt that certain organelles, such as centrioles and kinetosomes, have great morphogenetic potencies, and others, such as chloroplasts and kinetoplasts, are self-dependent, it may well be that the endoplasmic reticulum is more important than any of these in morphogenesis and cellular differentiation. With its intimate association with the nucleus of the cell on one hand and with the cell surface on the other, the membranes of the endoplasmic reticulum seem to provide a structural basis for the enactment of directions from the nucleus and for their modulation by the environment.

In protozoa as in other cells both rough- and smooth-profiled endoplasmic reticulum are present. It is of interest here that specimens of *Paramecium aurelia* were able to survive a 12-hour treatment with ribonuclease which removed most of their pyronin-staining material and virtually all their ribonucleoprotein particles visible by electron microscopy (Jurand *et al.*, 1962). The mitochondria and endoplasmic reticulum were not affected by the treatment. The ribonucleoprotein particles had not been regenerated after 56 hours in culture at 25°C., by which time most of the paramecia had divided once, but they had been regenerated after 72 hours, the ciliates by this time having undergone two fissions.

Endoplasmic reliculum is active in cell division and participates in the formation of new cell wall substance in dividing plant cells (cf. Porter, Chapter 9 in Volume II). It plays a somewhat similar role in the reproduction by schizogony of some of the parasitic sporozoa. In the erythrocytic stages of malaria the parasite cytoplasm is separated from that of the host cell by a double membrane. As the parasite grows its nucleus first enlarges and then undergoes a series of divisions to form a number of daughter nuclei (4 to 20 depending on the species). As nuclear division is nearing completion each nucleus becomes surrounded by dense cytoplasm contained within a double membrane (Rudzinska and Trager, 1961). These double membranes are not formed by ingrowth of the parent cell wall nor are they directly derived from

it. They seem to be produced *de novo*, probably from endoplasmic reticulum. In this way there is ultimately formed a group of uninucleate daughter cells, the merozoites, each having its own double-membraned wall, and all lying within a sac formed by the double-membraned wall of the now spent parent cell (Fig. 23). A similar method of daughter cell formation occurs in the related intracellular *Toxoplasma* (Gavin *et al.*, 1962).

There is some evidence (previously discussed) that at least two types of protozoan organelles, trichocysts and kinetosomes, arise from

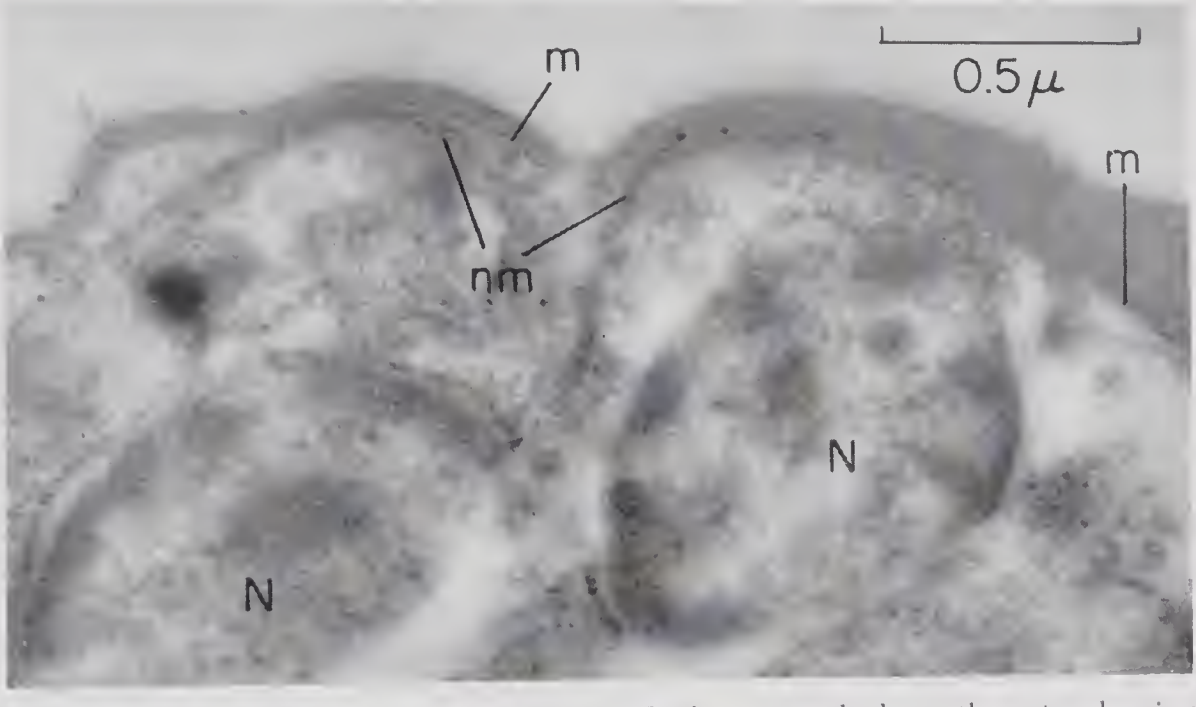

FIG. 23. Section through *Plasmodium lophurae* in duck erythrocyte showing formation of new membranes (*nm*) during schizogony. *m*, Plasma membrane of parasite; *N*, nucleus. Electron micrograph. From Rudzinska and Trager (1961).

simple vesicles not readily distinguished from other vesicles of the endoplasmic reticulum. Additional, more definitive evidence for this view is provided in a detailed electron microscope study of dividing paramecia made available to me before publication through the kindness of Dr. C. F. Ehret (Ehret and de Haller, 1963). The kinetosomes originate near the surface and their formation must be somehow controlled by existing kinetosomes. Trichocysts originate deep in the endoplasm, and what controls their formation has not yet been guessed at. If we consider the nuclear membrane as the constant portion of the endoplasmic reticulum, then the distinctions between possible origin of mitochondria from the nuclear membrane, or from vesicles in the endoplasmic reticulum, or from the surface membrane, become relatively unimportant.

More certain is the origin of the Golgi bodies (dictyosomes) and parabasal bodies of protozoa from the endoplasmic reticulum (see Dalton, Chapter 8 in Volume II of this treatise). The Golgi material in protozoa as in other kinds of cells consists of piles of smooth membranes joined in pairs to form sacs. Dictyosomes resemble those of other invertebrates and are usually more or less disk-shaped. They have been found in representatives of all the main groups of protozoa, although

FIG. 24. Electron micrograph of transverse section of parabasal body of *Trichonympha* showing the parabasal filament (*pf*) with the parabasal sacs (*ps*). Note inflation of the sacs (toward top of picture). Magnification: × 75,000. From Grimstone (1959).

most ciliates have no Golgi material (Grimstone, 1961). Parabasal bodies, found only in some groups of flagellates, are generally cylindrical and are attached to a centriole by a parabasal filament. This connection to the centriole is of interest in view of the frequent association of Golgi body with the centriole in other types of cells. Parabasal bodies always lie close to the nucleus. Their mode of reproduction is not clear. There is no good evidence that they divide (Kirby, 1944), and it seems most likely that they are formed anew. In some flagellates an old parabasal is carried to one daughter and a new one is formed in the other.

In the numerous parabasals of *Trichonympha* (Fig. 24) the sacs

farthest from the parabasal filament are inflated and seem to be pinched off as secretion vesicles in the adjacent cytoplasm (Grimstone, 1959). If the flagellates are starved the parabasal sacs decrease sharply in number until after 3 days only a few of the inflated sacs remain (Fig. 25). At the same time certain granular cytoplasmic membranes disappear completely. Upon feeding, the granular membranes and new

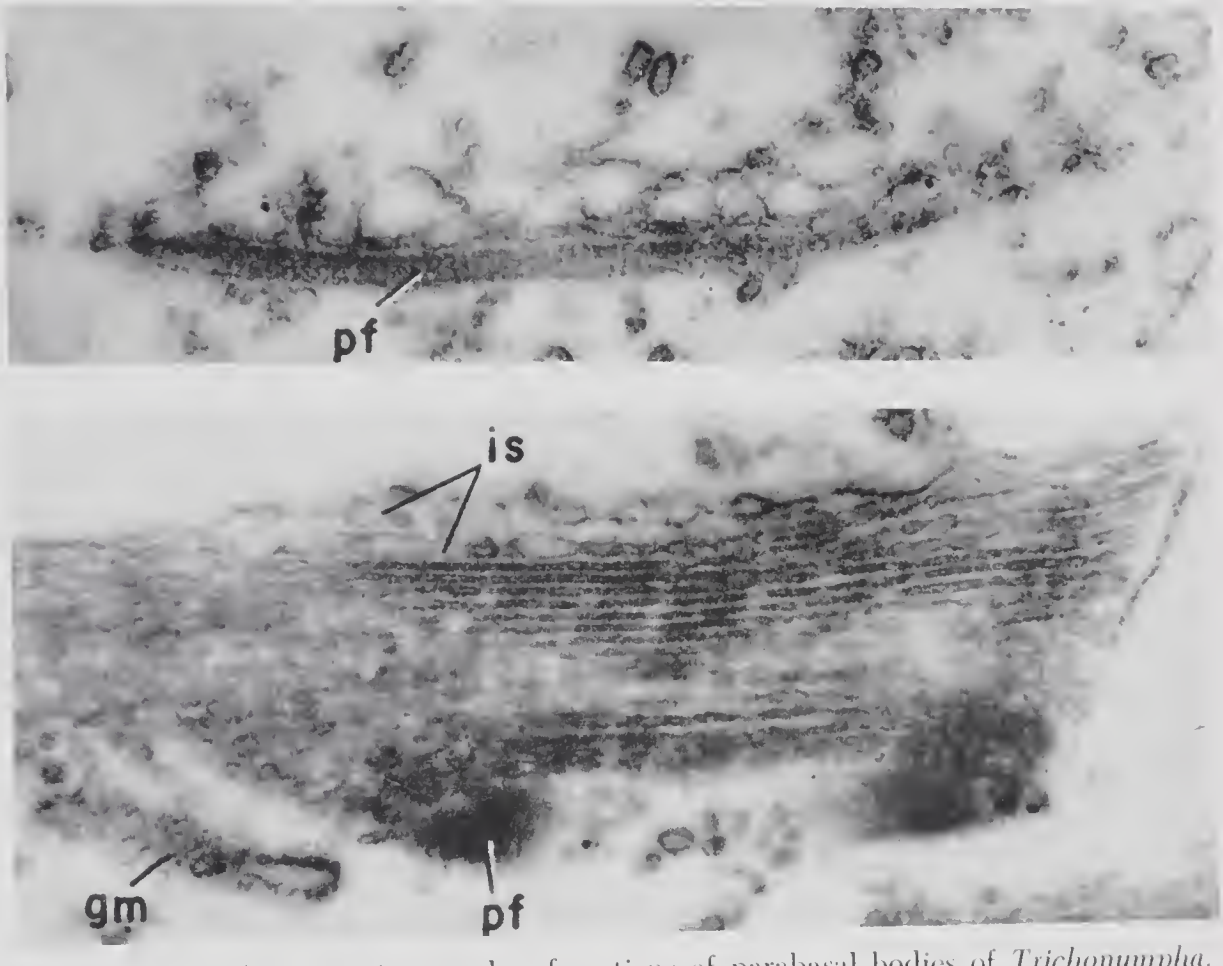

FIG. 25. Electron micrographs of sections of parabasal bodies of *Trichonympha*. Upper: after 3 days' starvation. Only the filament (*pf*) and a few inflated sacs remain. Lower: 2 days after refeeding, following 3 days' starvation. A pile of 17 sacs is present, of which the most distal are beginning to inflate (*is*). Note granular membrane (*gm*). Magnification: × 49,000. From Grimstone (1959).

uninflated parabasal sacs reappear, suggesting possible derivation of the latter from the former. Also supporting this suggestion is some indication that the granular membranes in turn are derived from the nucleus, which as always is close to the parabasals. The parabasal body could be looked upon as being normally in a state of dynamic turnover, new sacs being derived from granular membranes, becoming inflated with secretory products as they move distally, and finally being shed into the cytoplasm as vesicles.

A more detailed experimental study of nutrition in relation to the large parabasal bodies of *Trichonympha* would be very desirable, but this is difficult because flagellates of this genus have not been grown in culture. A similar study of the simpler but still well-defined and conspicuous parabasal body of the trichomonads, many of which can be readily cultured (see for example Lee *et al.*, 1962) should be just as informative and easier to do.

X. Morphogenesis; Life Cycles

Cytodifferentiation involves activities of both the nucleus and the cytoplasm; neither can be considered more important than the other. We tend to think of the nucleus as supplying the initiative and directive force, and recent work with messenger RNA strongly supports this idea. But certainly messages get back from the cytoplasm to the nucleus—else how would the nucleus of a *Stentor* with its head cut off know that it should get the cytoplasm to work making a new one? The cytoplasm, moreover, has a highly patterned organization. This has long been known in many kinds of eggs, and it is beautifully illustrated in the large ciliate *Stentor* (Tartar, 1961). Cutting and grafting experiments show that every part of the cortex of this unicellular animal has an anteroposterior and a left-right polarization. Moreover, the place of formation of a new mouth before cell division and the position of the fission line are fixed long before these become visible. The differentiation in ciliates of a new mouth and other organelles preparatory to binary fission is a dramatic example of one of the two main kinds of morphogenetic events occurring among protozoa—that associated with reproduction (see Trager, 1963). The formation of a new mouth in *Stentor* involves thousands of basal bodies, organelles whose synthetic activities we have already considered in some detail. But there are subtler activities of the cytoplasm. In *Stentor*, as in multicellular organisms, formed parts inhibit neoformation of their like. Thus, although removal of the mouth initiates its complete regeneration, if a well-formed oral anlage is grafted onto a nondifferentiating stentor with a mouth, the anlage is resorbed. These facts give rise to two questions, neither of which has been answered satisfactorily. (1) What is the nature of the inhibitory principle which ordinarily prevents a stentor from having two mouths? (2) How is this inhibition suspended to permit development of a second mouth preparatory to cell division?

The second main category of morphogenetic events occurring among protozoa involves processes of reorganization, often not accompanied by reproduction. Here belong the changes which take place during the

life cycle of protozoa, including encystment and excystment, sexual processes, various structural and physiological adaptations, as well as restorative events elicited by environmental stimuli. A simple life cycle is illustrated by certain free-living ciliates which can exist either as small forms with a small mouth (microstomes), which feed on bacteria, or as large forms with a relatively much larger mouth (macrostomes), which feed on other ciliates (Fig. 26) (Williams, 1960). One such ciliate, *Tetrahymena patula*, has recently been studied in axenic culture.

FIG. 26. *Tetrahymena patula*. Microstome on left, macrostome on right. Photomicrographs of silver preparations. *UM*, Undulating membrane; *M*, location of the three membranelles. From Williams (1960).

Both temperature and pH affected the proportion of macrostomes, which was always greatest toward the end of the growth cycle. During logarithmic multiplication only microstomes were present. In the stationary phase, at pH 7.0 and a temperature of 20° C., 100% of the organisms changed to macrostomes, whereas at 25° C. only 50% became macrostomes (Stone, 1963). In the conversion from microstome to macrostome the old oral apparatus is not directly transformed into the new; instead the old oral apparatus is resorbed and replaced by a new mouth formed from a field of kinetosomes arising just back of the old mouth. Cell division is not involved here, but in the reverse change from macro-

stome to microstome one macrostome always gives rise to two microstomes.

Another reversible differentiation equally accessible to experimental study is the process of encystment. Protozoan cysts may be highly differentiated structures, even though the organism within may lose most of its extranuclear organelles, only to regain them before excystment. Profound physiological as well as morphological changes occur. When the respiration of individual ciliates (*Colpoda cucullus*) was measured during encystment and excystment, it was found that the ciliate secreted a delicate cyst membrane before there was any marked decrease in respiratory rate or rate of pulsation of the contractile vacuole (Pigon and Edström, 1961). The respiratory rate then fell within 24 hours from an average of 11.3×10^{-5} µl. O_2 per hour to only 1.3, at which low level it remained at least 4 weeks. When such a cyst was put in hay infusion, its respiration increased within an hour or two and its contractile vacuole reappeared. Excystment occurred in 2–3 hours, and the respiratory rate reached its preencystment level after a further 1 or 2 hours.

A third reversible differentiating system among protozoa is the amebo-flagellate transformation (Hollande, 1942). The cysts of species exhibiting this transformation always give rise to amebae; these may then multiply as amebae indefinitely. Under certain conditions, however, which vary with the species and are as yet imperfectly understood, the amebae transform into flagellates. In *Tetramitus rostratus*, when an ameba is about to change to a flagellate two basal bodies appear and migrate rapidly to the posterior pole of the ameba; each produces two flagella. The pseudopods of the ameba retract, its body becomes conical, a cytostome forms at the flagellated end which is now anterior, and the flagellate swims away. The change takes only 30 minutes, but the reverse change is slower. In another species, *Naegleria gruberi*, Willmer (1956) has shown that certain ions, notably Mg^{++}, are specifically effective in suppressing the change from ameba to flagellate, a change which will occur in nonelectrolyte solutions even as concentrated as *M*/5. Very recent work with *Tetramitus* (Outka, 1962) indicates that control of transformation in this species is more complex than had been supposed and is in part a function of population density. The development of methods for axenic culture (Brent, 1954) and of defined culture media (Balamuth and Outka, 1962), together with the application of electron microscopy and techniques for cell fractionation and enzymatic assays should permit a detailed study of what really happens in this remarkable transformation.

Perhaps still more remarkable are the transformations, especially

in metabolic patterns, found in the life cycles of some of the hemoflagellates. These have already been discussed in relation to the activities of the kinetoplast in mitochondrial formation. A change simpler than but analogous to the change occurring when the African mammalian trypanosomes acquire infectivity has been studied in some detail in a trypanosome of frogs, *Trypansoma mega*. This parasite grows in culture as a crithidia, corresponding to stages in an invertebrate vector. In the blood of the frog, however, it exists as a larger trypanosome form with a conspicuous undulating membrane. Steinert (1958) found that a small proportion of trypanosomes would appear in crithidial cultures exposed to a high concentration of serum. It turned out that the active substance in serum was not a protein, as expected, but urea. Urea at a concentration of only 0.016 *M* could bring about the appearance *in vitro* of typical blood stream trypanosomes of this species. The percentage of flagellates transformed by urea was a function of the age of the culture. During the phase of logarithmic increase urea had no effect. In the stationary phase, reached after 6 days at 25°C., up to half of the crithidia changed into trypanosomes in the presence of urea. Here we have in a microorganism the phenomenon called "competence" by embryologists; only in stationary phase do the flagellates become competent to transform into trypanosomes under the influence of urea. How urea does this remains to be discovered. Urea added to a competent culture stops the uptake of tritiated thymidine by those cells which transform, but not by those which remain as crithidia (Steinert and Steinert, 1960). Whether this is cause or effect of the transformation we cannot yet say.

An insect hormone, ecdysone, has now been shown to have morphogenetic effects on certain protozoa. The many species of flagellates in the hindgut of the wood-feeding roach *Cryptocercus* ordinarily reproduce by binary fission accompanied by mitotic nuclear division, but they all exhibit sexual phenoma associated with the molting of their host (Cleveland, 1956a). In the genus *Trichonympha*, for example, about 6 days before the roach is going to molt the asexual, haploid, flagellated forms round up, produce a thick wall and lose most of their extranuclear organelles. Within the cyst haploid gametogenesis occurs with the formation of one male and one female gamete, distinguishable by the paler staining of the chromatin of the female. New complete sets of organelles are then formed by the activities of the centrioles in each gamete, and the gametes excyst just after the roach has molted. Sexual union follows with penetration of a male gamete into a female. The male loses all its extranuclear organelles including

its centrioles. The two nuclei then fuse to form a zygote which undergoes typical two-division meiosis to produce four haploid asexual cells. The whole process is complete 2 days after molting of the roach.

The details of gametogenesis, fertilization, and meiosis, as well as the timing of these processes, differ among the different genera of flagellates. All the changes, however, can be brought about by the injection into the host roach of crystalline ecdysone (Cleveland *et al.*, 1960). This is true even if the hormone is injected into an adult roach or an intermolt nymph in such dosage that the host itself does not undergo ecdysis. Some genera of the flagellates begin gametogenesis within only 3 hours after the injection of 100 units (0.75 mg.) of ecdysone. Ecdysone may affect directly the chromosomes of insect cells (Clever and Karlson, 1960), and perhaps its effect on the cytoplasm of protozoa is likewise exerted via the nucleus.

The same may well be true of the several other relatively simple substances having morphogenetic effects, as urea in *Trypanosoma mega.* Whatever the situation ultimately turns out to be, there can be no doubt that much more must be learned concerning the intimate nature of the cytoplasmic milieu—the structured medium within which the nucleus and the cytoplasmic organelles interact to produce specialized substances and forms. In studies directed to this end, protozoa, with their diverse cellular structures and their amenability to experimental work, will continue to be valuable material.

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CHAPTER 3

The Fungi

By JOHN R. RAPER[1] and KARL ESSER[2,3]

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[3] The senior author is responsible for Sections II, III, and VI and the translation of Sections IV and V, which were written in German by the junior author.

I. Introduction

The fungi are generally and broadly defined as achlorophyllous, thallus plants. This definition, however, embraces three large and diverse groups, the bacteria, the Myxomycetes, and the "true" fungi, between which common features are limited to the inclusive, defining criteria and among which relationships are completely obscure—as obscure, in fact, as is the relationship of the entire group to the plant and animal kingdoms (Martin, 1936, 1941; Rogers, 1948). For present purposes, the definition of fungi is more narrowly construed to include only those forms that possess both organized, membrane-enclosed nuclei (lacking in the bacteria) and definitive cell walls (absent in the Myxomycetes). Two universal features of the fungi prescribe the biological niche that they occupy and determine the major aspects of their biological activity: (1) their lack of chlorophyll and their inability to photosynthesize make them nutritionally dependent upon other living organisms, directly as parasites or indirectly as saprophytes; and (2) their simple, delicate, and exposed thalli dictate an essentially aquatic habitat during at least the critical, vegetative period of growth. A third feature, common to a vast majority of forms, accounts for the characteristic fungal mode of growth: the organization of the vegetative thallus as a system of radiating, separate, branching filaments (hyphae) that grow by apical extension.

Even so, the fungi constitute a remarkably diverse assemblage in respect to habitat and substrate, morphological characteristics, developmental patterns and life cycles, and biochemical and biosynthetic capabilities. The diversification of the fungi follows from the fact that many features and processes vary widely and independently and are frequently expressed as variants that do not occur in other groups of organisms.

The present chapter combines a brief consideration of the general characteristics of the fungi with a somewhat more detailed account of those aspects peculiar to the fungi and of those features that have prompted the use of fungi for the elucidation of basic biological phenomena. Only occasional references to the original literature will be given in the sections dealing with general considerations, since these sections unblushingly summarize the relevant information from the standard mycological texts (Gäumann, 1926, 1949; Gäumann-Dodge, 1928; Gwynne-Vaughan and Barnes, 1937; Wolf and Wolf, 1947; Alexopoulos, 1962; Bessey, 1950; Sparrow, 1943, 1961). More complete bibliographic citations, however, will be provided for those sections that

deal with the more important unique features of the fungi and those areas of study that have special relevance to basic phenomena.

II. Morphology

Two basic and distinet phases may be reeognized in the developmental histories of practieally all fungi: (1) the vegetative phase consists, in a majority of cases, of a hyphal system, or myeelium, which typically shows little or no structural or functional differentiation, and (2) the reproductive phase relates to the produetion of asexual, sexual, or asexual and sexual reproduetive elements that are often highly differentiated and may range in size from mieroseopie to large (mushrooms, puffballs, etc.) and in durability from ephemeral to perennial. Reproduction may begin early and thus largely coincide with the vegetative phase; in other cases, the reproductive phase may be variously delayed to oceur late in vegetative growth or even to terminate all vegetative aetivity.

A. The Vegetative Phase

Three aspeets of the vegetative phase deserve brief review: (1) the cellular organization of the mycelium (or clone), of which the major variants are loosely eorrelated either with phylogenetie groupings or with certain functional modifieations; (2) the pattern of vegetative propagation, a charaeteristie mode of growth that follows from the structural features of the myeelium; and (3) the detailed morphology of the mycelium as determined by anatomical variations at the level of the constituent cells.

1. Cellular Organization

The great majority of fungal species are organized as radiating systems of distinct hyphae, i.e., as myeelia; two exceptional groups, however, will be considered below. The hypha grows by the simultaneous extension of the apex and the formation of lateral branehes, a fraction of which rapidly assumes the radial orientation and the apieal mode of growth of the parent hyphae. The basie types of hyphal structure and cellular organization, however, are evident in the eoenoeytie mycelium of the Phycomycetes and in the septate myeelium of the higher fungi, the Aseomycetes and Basidiomycetes.

a. Coenocytic organization. The hyphae of the Phycomyeetes are typically coenocytic, with large numbers of nuclei randomly distributed throughout a common eytoplasm and with septa formed only in aged hyphae or to delimit reproductive elements. The detailed hyphal morphology varies enormously, usually in loose eorrelation with different

phylogenetic groups and with specific habitat. In the simpler aquatic forms, the entire mycelium may consist of a single, multinucleate "cell," which at maturity is transformed *in toto* into a reproductive body, either asexual or sexual (see below). At the other extreme, and epitomized by the "black bread molds," the Mucorales, the vegetative body consists of an extensively branched mycelium that is capable of indefinite growth and upon which are borne highly differentiated reproductive elements. The vegetative mycelium of many phycomycetous forms may be differentiated obligatorily or facultatively into two portions: (1) a rhizoidal system consisting of thin, highly branched hyphae that are sharply tapered toward their apexes and are completely submerged within the substrate; and (2) an aerial or aquatic portion consisting of stouter, longer, and less highly branched hyphae. The development of a rhizoidal system is sometimes determined by—and in other cases, independent of—the specific mode of culture: upon an agar medium, the zoospore of a typical biflagellate "water mold," *Achlya*, develops into a vegetative mycelium that lacks a rhizoidal system, whereas upon a particulate substrate, rhizoids are formed; the zoospore of a typical uniflagellate "water mold," *Allomyces*, forms a well-developed rhizoidal component under either condition. In most aquatic and terrestrial mycelial forms, growth is indefinite upon a uniform, continuous substrate, while growth beyond the substrate is characteristically restricted upon particulate substrates. Filamentous Phycomycetes that are parasitic upon algae, other fungi, higher plants, and occasionally upon animals, range from very restricted—sometimes in a single host cell—to indeterminate ramification within the host tissue.

b. "Unicellular" coenocytic organization. Among the more primitive Phycomycetes are many small, superficially amycelial forms that are primarily parasitic on other fungi, particularly the larger aquatic Phycomycetes, algae, protozoa, and higher plants. The vegetative thalli of these simple forms, which become multinucleated during growth, are coenocytic and differ from the above primarily in the lack of competence for extensive growth.

c. Septate organization. With the exception of the unicellular yeasts (see below), the higher fungi, i.e., the Ascomycetes, Basidiomycetes, and Fungi Imperfecti, are all but universally mycelial and constituted of septate hyphae. The size of individual cells varies tremendously both in breadth and in length, and other features are equally variable. The number of nuclei per cell ranges from one to as many as a hundred and is relatively constant within a species and among closely related species. Thus, the less highly specialized Ascomycetes, the Protoascomycetes,

typically have uninucleate cells; the more highly developed members of this group, the Euascomycetes, most commonly possess cells having a large and indefinite number of nuclei; the Basidiomycetes characteristically have uninucleate cells in the haplo- or homokaryotic phase and binucleate cells in the dikaryotic phase. There are, however, many exceptions to this general pattern. In actuality, the contiguous, multinucleated cells of many Ascomycetes approach a functional coenocytic organization, since, in addition to the large number of nuclei populating a common cytoplasm within each cell, there is cytoplasmic continuity between adjacent cells via large, simple septal pores (see Section II, C, 1). Hyphal branches arise as lateral cellular outgrowths, usually of subterminal cells; they may occur at any point along the length of the cell, although most commonly they originate just back of septa. The extent of growth of lateral branches depends upon their position relative to existing hyphae: near the apexes of hyphae, the laterals often become radially oriented and assume the habit of growth of radiate, major hyphae; in inferior positions, the growth of laterals is usually sharply restricted.

d. Unicellular (clonal) organization. A true unicellular and clonal habit, the only basic departure from a mycelial organization to be found among the fungi, occurs in the yeasts of the Ascomycetes, in the haplophase of the smuts of the Basidiomycetes, and in a few scattered forms under special conditions, e.g., certain of the "black bread molds" under anaerobiosis. Even in the yeasts, however, the unicellular habit appears not to be rigidly fixed, and many species grow either as clones of separate cells or as mycelia depending upon such factors as aeration, concentration of nutrients, etc. Among unicellular forms, cellular multiplication may occur by budding (in a majority of the yeasts and in the haplophase of the smuts) or by transverse septation (in a few yeasts). In the budding yeasts and in the smuts, the separation from the mother cell of developing buds is variable, and in many cases, delayed separation leads to the formation of clumps of a few to hundreds of cells; in the fission yeasts, the rounding of the newly formed end walls by turgor pressure early separates sister cells. Upon semisolid media, vegetative growth leads, in budding and in fission forms, to the formation of colonies which vary widely—the expression of the summed characteristics of the constituent cells—in size, color, texture, and margin.

2. *The Dynamics of Fungal Growth*

The dynamics of growth of unicellular forms differs in no essential manner from that of other unicellular microorganisms. The typical

growth curve consisting of lag phase, exponential multiplication, and senescence applies, and it requires no addition here to the numerous descriptions already available (Thimann, 1955; Oginsky and Umbreit, 1955; Cook, 1958).

The dynamics of mycelial growth, however, is far less well known and deserves consideration, since a basic pattern of growth is common to a majority of the fungi yet is peculiar to the fungi as a group—with the possible exception of a few filamentous algae in which growth is restricted to apical elongation.

The manner in which new structural units are added is best seen in septate forms, although the same principles should apply equally well to coenocytic forms. In the apical cell, the extreme tip is densely packed with protoplasm, and there is a marked gradient in protoplasmic density along the cell to its base (Girbardt, 1960). Elongation of the cell by extension and the deposition of new wall materials occur only in the terminal region. Following cellular elongation, septation near the center of the cell divides it into two daughters. The apical daughter cell repeats the growth-division cycle; the subterminal cell, however, may or may not participate significantly in further growth by the formation of a lateral branch endowed with the competence for apical growth. Occasional septation of nonterminal cells may occur, but a very high percentage of all new cells is laid down by apical growth. This restriction stands in marked contrast to the competence of all cells of a clone of a unicellular form to produce two equal daughters, and this distinction is reflected in the unique dynamics of mycelial growth.

The mycelial growth curve still retains the major features of the typical unicellular curve: lag phase, "grand" period of growth, and senescent decline. The significant feature of the curve is its specific nature during the "grand" period of growth; the functions that best define the accumulation of newly incorporated living material, however, are easily reconciled with the basic features of the mycelium (Emerson, 1950). The following features are relevant, whether they be applied to the cells of a septate mycelium or to unit segments of a coenocytic mycelium: (1) the radiate growth of individual hyphae is a linear function of time (Ryan *et al.*, 1943); (2) the growth of lateral branches, however, is restricted according to their position and to the prior presence of preexisting hyphae in the immediate vicinity, and consequently (3) the conversion of lateral branches to maintain a fixed and characteristic hyphal density at the level of growing tips. The precise shape of the growth curve, during a period when other conditions are not limiting, thus depends upon the spatial restrictions imposed

upon the system. (1) If the mycelium is confined to a narrow strip of substrate, as in a growth tube, the increase in material as measured by dry weight would be expected to be constant, and the total to be a linear function of length—hence of time. (2) If the mycelium is permitted growth in two dimensions, as from the center of a shallow layer of medium in a culture dish, the increase in material should be a linear function of time, and the total accumulation should be a squared function of time. (3) If the mycelium is not limited spatially and is free to grow in three dimensions, as in a deep layer of liquid medium, the mycelium early assumes a spherical shape, and its rate of increase should be a squared function and the total a cubed function of its radius—hence of time. The constancy of linear or radial growth has been incidentally confirmed in numerous studies with fungi of many different types (Cochrane, 1958). The expected characteristics of three-dimensional growth under nonlimiting conditions have been demonstrated in *Neurospora* by Emerson (1950), and there is no reason to question the universal applicability of his findings.

3. *Mycelial Morphology vs. Cellular Morphology*

The preceding sections have emphasized the similarities and common features of mycelia of various types. If we consider only the septate types, however, there is a remarkable range of mycelial morphologies in view of the simple common structure of branched filaments consisting of cylindrical cells joined end-to-end. Such variation reflects the summed anatomical and developmental details of the constituent cells, which can and do vary in a number of important ways: length, diameter, place and mode of proliferation after septation, i.e., branching and angle of branching, relative growth of branches *vis-à-vis* that of radiate hyphae, and the cellular density that may be achieved. Independent variation of these several characteristics can well account for the wide range of mycelial morphologies that occur, but the detailed correlation implicit in this causal relationship has been, so far as the authors are aware, the primary objective of no critical work, although it is apparent on occasion in published reports (Murray and Srb, 1962). A comparative survey of cellular morphology in fungi would have relevance, however, to the eventual understanding of the regulation of cellular, hence mycelial, characters.

That cellular morphology in the normal mycelium is under elaborate control is indicated by observations—incidental to other studies—on morphological mutations in the Basidiomycete *Schizophyllum* (Raper *et al.*, unpublished). The normal, wild-type mycelium, constituted of

cells of reasonably uniform size and constant features of branching, etc., is subject to a large number of very striking alterations, each the result of a recessive mutation of a distinct, single locus. These mutant mycelia are each clearly distinguishable, both macroscopically and microscopically, from wild type and from each other. A number of the better known deviations from normal, expressed as anatomical anomalies at the cellular level, are listed in Table I and two of these and the normal form are illustrated in Fig. 1.

The mycelial alterations illustrated here represent only a fraction—the more striking examples, admittedly—of the sample that has been isolated incidental to the procurement of biochemical mutations, the characterization of heterokaryons, etc. Viewed from the reasonable assumption that each abnormality results from the impairment of a single function required for normal development, the number of points in the normal control that are subject to mishap is perhaps less surprising than the expression of some of the mishaps themselves. For example, the nature of the function, the impairment of which leads to the outlandishly bizarre mutant *puff* with its complex of altered characters, is entirely unknown; it has been established, however, that a mutation at either of two unlinked loci results in the expression of puff morphology (Raper and Miles, 1958).

Such observations serve both to show the basic relationship between cellular and mycelial morphology and to suggest a promising field of study for the eventual integration of genetic, biochemical, and developmental phenomena.

4. *Specialized Vegetative Structures*

Although the vegetative mycelium is typically undifferentiated, two types of relatively massive, compact vegetative structures to be found in numerous higher fungi deserve brief mention.

a. Sclerotia. Local and extensive proliferation of hyphae may result in the formation of a compact knot of mycelium, the sclerotium, which, because of the mutual pressure exerted by its constituent cells, assumes a pseudoparenchymatous inner structure. The inner cells contain large reserves of food materials, and the outer cells are often thickened to form a protective, cortical layer. Tough and resistant, sclerotia serve to carry the fungus through periods of unfavorable conditions; with the return of favorable conditions, growth is resumed in the reestablishment of a vegetative mycelium or in the production of characteristic asexual or sexual reproductive elements.

Also frequently associated with reproductive bodies, particularly

TABLE I

MYCELIAL MORPHOLOGY AND ANATOMICAL FEATURES OF COMPONENT HYPHAE OF MUTANT STRAINS OF *Schizophyllum commune* AS COMPARED TO NORMAL[a]

Strain	Mycelial morphology			Cellular morphology			
	Extent of growth	Aerial component	Density	Length	Diameter	Branching and angle	
Puff	—	—	—	(main) 0	+	—	obtuse
				(lateral) — —	+	++ (dichotomous)	
Fir	— —	0	++	—	0	++	0
Feather	— —	0	+	—	0	++	acute
Thin	+	— —	—	+	+	—	0
Streak	0	—	0	0	0	+	acute

[a] Symbols +, —, and 0 indicate, respectively, greater than, less than, or no different from, normal.

among the Ascomycetes, are compacted masses of vegetative mycelium known as stromata. These amorphous aggregates, indefinite in size and shape, serve as a base upon which or within which reproductive structures are formed.

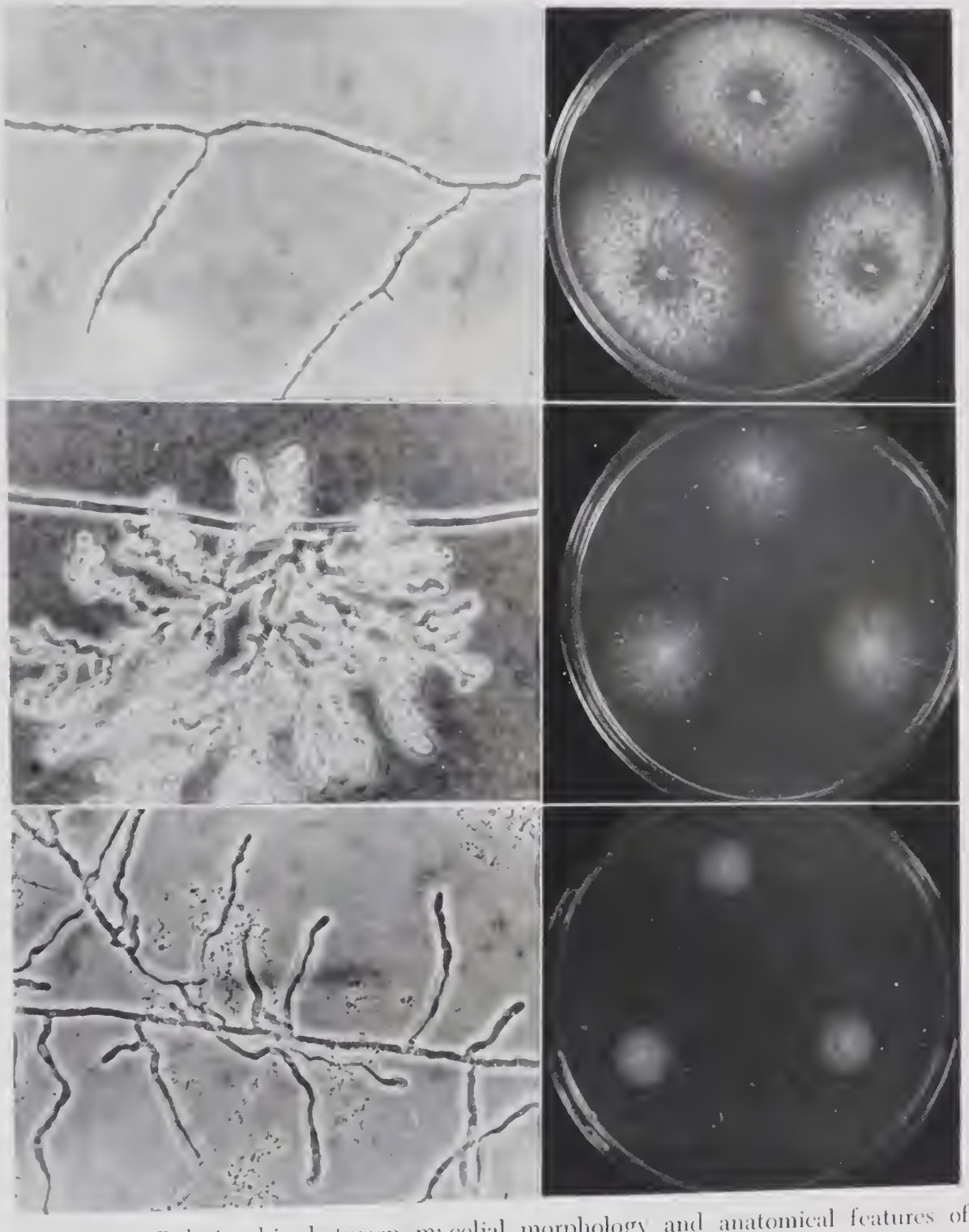

FIG. 1. Relationship between mycelial morphology and anatomical features of component hyphae in wild and mutant strains of the Basidiomycete *Schizophyllum commune*. Above, wild type; center, *puff*; below, *fir*.

b. Rhizomorphs. In numerous wood-rotting and soil-inhabiting Basidiomyeetes, hyphae may beeome laterally compaeted to form hard, dense strands, rhizomorphs, that may extend outward for many feet from the parental myeelium. The outer layers of cells form a protective sheath about the inner pseudoparenchymatous tissue, whieh in turn surrounds a eontinuous lumen. Rhizomorphs serve the dual funetion of the transport of food materials and the spread of the myeelium to additional substrate areas, e.g., the roots of trees upon whieh the fungus is parasitic. For recent studies on rhizomorphs see Garrett (1956a, b) and Snider (1957).

B. The Reproductive Phase

The reproductive phase in praetieally all fungi reaches a far higher level of differentiation and specialization than occurs in the vegetative phase. Furthermore, the reproduetive phase is far more distinctive of the various classes and subclasses of fungi than is the vegetative, nutrient-gathering mycelial phase. In addition to strietly vegetative means of propagation, usually the result of the mechanieal dismemberment of the myeelium, most fungi produee eharaeteristie, specialized reproductive elements, asexual or sexual, and it is primarily upon these features that the systems of taxonomy of the fungi are based. The types of sexual reproduetive struetures serve to distinguish elasses, i.e., Phyeomyeetes, Aseomyeetes, and Basidiomyeetes—and the Fungi Imperfecti, which laek sexual reproduetion. The details of sexual struetures differentiate lesser groupings to the level of orders and families, while asexual reproductive features most commonly distinguish lower taxa at the level of genera and speeies.

Spores of many types are commonly the ultimate means of reproduetion. Spores, however, eonstitute only the end products of the reproductive phase, and the various spore types will be considered here in conneetion with the several means of their produetion. For our present purpose, a brief summary of the major types of speeialization eorrelated with reproduction will suffiee.

1. Asexual Reproduction

The distinction between asexual reproduction and certain types of vegetative propagation is rather tenuous, and some of the spore types considered below are classified as vegetative elements by other authors (Hawker, 1957). Asexual reproduction is here considered to include those means of multiplication that involve speeialized structures not originating in or associated with plasmogamy, karyogamy, and meiosis, i.e., the sexual process.

Asexual reproduction is typically achieved by means of spores, the more important categories of which, together with the means of their production, are characterized as follows and are illustrated in Fig. 2.

a. Sporangiospores. The asexual spores of most of the Phycomycetes are produced in sporangia that originate either (1) by the swelling and differentiation of hyphal tips, e.g., "water molds," or (2) *de novo* at the end of specialized hyphal branches, sporangiophores, e.g., "black bread

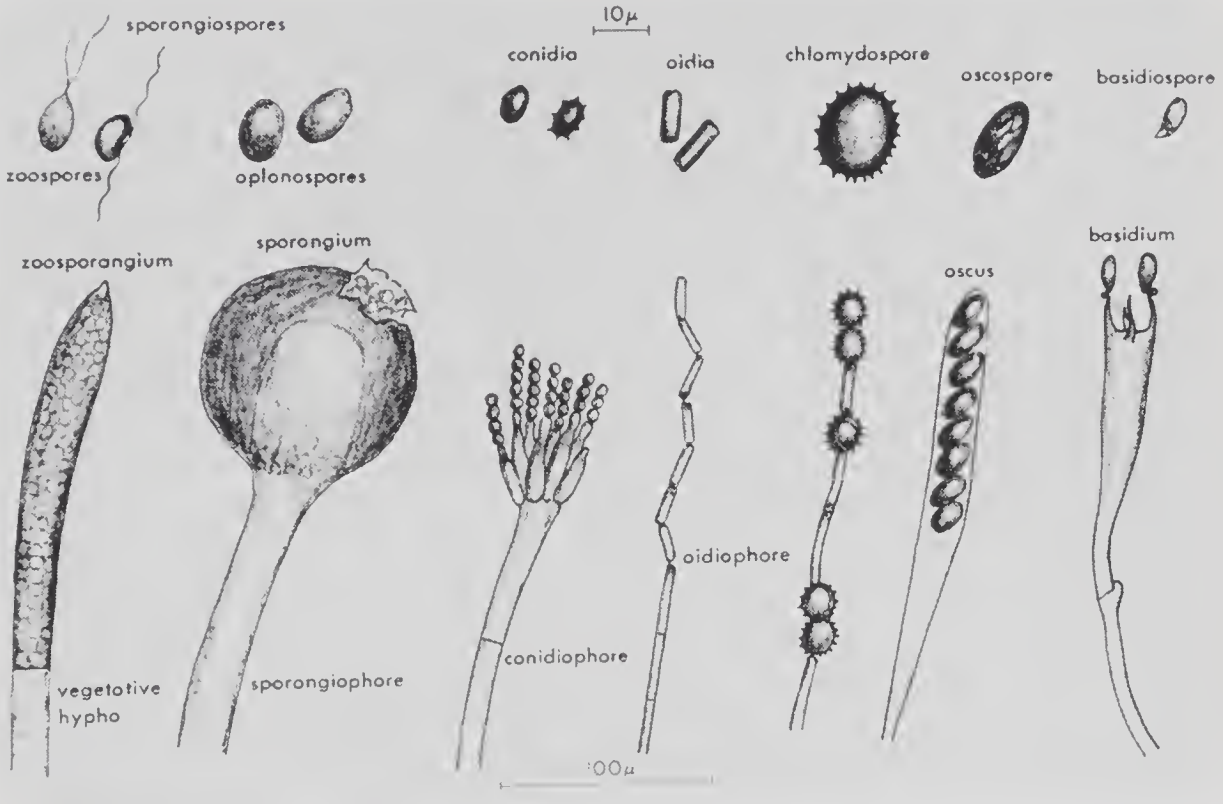

FIG. 2. Representative spore forms in fungi. The five types at the left are asexual; the two at the right are the final products of sexual reproduction in the Ascomycetes and Basidiomycetes, respectively. (From Raper, *in* "McGraw-Hill Encyclopedia of Science and Technology," Vol. 5. McGraw-Hill, New York, 1960. Used by permission.)

molds." The developmental pattern is much the same in either case. Protoplasm, including large numbers of nuclei, flows into the tip of the hypha or sporangiophore and results in a slight to globose enlargement of the tip; a septum is then laid down to delimit the developing sporangium from the coenocytic, vegetative hypha. Cleavage of the protoplasm is then initiated from the central vacuole and proceeds centrifugally to cut the protoplasm into small blocks, each containing one to a few nuclei. Each uninucleate or multinucleate block of protoplasm then differentiates as a spore. The final stage in differentiation depends

upon the nature of the spore and involves either the elaboration of a locomotor apparatus in those forms with motile spores, plano- or zoospores, or the deposition of an external cell wall for nonmotile spores, aplanospores. Zoosporangia typically dehisce by means of one or more emergence papillae or an ephemeral emergence vesicle; the zoospores may either swim away immediately, or they may encyst at the mouth of the sporangium later to emerge and swim away. Aplanosporangia usually liberate their spores by the disintegration of the sporangial wall. An unusual variant of this latter behavior occurs in the dung-inhabiting form, *Pilobolus*: the entire sporangium is forcibly discharged toward the light for a distance of several feet by the specialized sporangiophore.

Three basic types of zoospores occur among the aquatic Phycomycetes. (1) Monoflagellated spores with the flagellum inserted in a posterior position characterize the monoflagellate series of Phycomycetes, which include most of the simple, amycelial forms and numerous filamentous forms such as *Allomyces*. The single posterior flagellum is of the whiplash type (Couch, 1941; Koch, 1958, 1961); a deep-staining "nuclear cap," now thought to be composed largely of ribosomes (Blondel and Turian, 1960), is present in most zoospores of this type (Hatch, 1935, 1938; Turian, 1955; Koch, 1958). (2) Monoflagellated spores with anterior flagella of the tinsel type occur in a single group of small, amycelial forms, the Hypochytridiaceae (Sparrow, 1943). (3) Biflagellated spores typify the biflagellate series of Phycomycetes, which, in parallel with the monoflagellate series, contains forms ranging from amycelial to extensively mycelial. The insertion of the flagella, one of which is whiplash, the other, tinsel, may be anterior at the time of release of the spore from the sporangium; following encystment and reemergence, however, the spore assumes the kidney shape characteristic of the entire series. The insertion of the flagella is now lateral, with one flagellum directed anteriorly (tinsel), the other, posteriorly (whiplash). In other cases, flagellar insertion is lateral from the beginning (Couch, 1941).

Aplanospores are simple in form and possess a definite wall, the outer surface of which may be either smooth or variously sculptured and marked.

b. Conidia. The characteristic asexual reproductive spore of the Ascomycetes and the Fungi Imperfecti is the conidium, a simple spore, singly borne upon cells of the vegetative mycelium or upon a specialized hyphal branch, the conidiophore, and typically enclosed within a multilayered wall. Conidia occur in the Phycomycetes only in a few advanced forms, in which multispored sporangia are progressively replaced by

small, few-spored sporangia and one-spored sporangia, i.e., conidia. Conidia also are produced by some species of Basidiomycetes, but it is in the Ascomycetes and the associated Fungi Imperfecti that conidia occur most commonly and in the widest range of variant forms.

Conidial production may be achieved by numerous means, of which the following are the better known: (1) as differentiated fragments of terminal hyphal branches, e.g., *Neurospora*; (2) as outgrowths from a vesicular conidiophore, e.g., *Cunninghamella*; (3) by successive terminal constriction of specialized cells, sterigmata, arranged on a vesicular conidiophore, e.g., *Aspergillus*, or located at the ends of a highly branched conidiophore, e.g., *Penicillium*; and (4) extruded singly from specialized cells, phialids. Conidiophores may be borne (*a*) singly and exposed upon the vegetative mycelium, (*b*) clustered together in groups, or (*c*) within differentiated structures, covered by a sheath of sterile vegetative hyphae. Conidia initially contain one nucleus to a few nuclei and are characteristically unicellular at formation; multinucleate conidia may later become multicellular by septation.

Conidia, dispersed to favorable conditions and substrate, each germinates by the formation of one or more germ tubes to reestablish the vegetative mycelium. Rarely, condiia may serve as spermatizing agents in sexual reproduction, e.g., *Neurospora*.

c. Oidia. In a number of forms scattered throughout the higher fungi, effective asexual reproduction is achieved by means of oidia. These are formed at the ends of vegetative hyphae as uniform, scarcely differentiated, hyphal fragments that separate easily. The number of included nuclei is of the order of 10–100. Germination, under proper conditions, occurs readily by the swelling of the oidium and initiation of growth at its ends to reestablish the vegetative mycelium. Oidia, like conidia, may serve as spermatizing agents in sexual reproduction, e.g., *Ascobolus*.

d. Chlamydospores. Heavy-walled chlamydospores occur in members of all major groups of fungi as specialized resistant elements either intercalated along vegetative hyphae or formed terminally on hyphae. The nuclear content of the chlamydospore corresponds to that of the vegetative cell from which the spore was formed and may vary from one to a few. The heavy walls of these spores are frequently sculptured or marked. Chlamydospores serve primarily as resistant elements to carry the vegetative mycelium through periods of unfavorable conditions. Germination is by rupture of the spore wall and the emergence of one or more germ tubes.

2. *Sexual Reproduction*

The sexual reproductive organs and the structures associated with the sexual reproductive process are the most distinctive features of the several groups of fungi. Throughout the Phycomycetes and the Protoascomycetes, the simple reproductive organs are borne exposed upon the vegetative mycelium. These organs, which consist of the sexual apparatuses and the products of their interaction, display a high degree of differentiation and will be characterized in Section III on life cycles and sexuality. Attention here will be directed toward the secondary structures that are associated with sexual reproduction. These are of two main types: the ascocarp and the basidiocarp of the Ascomycetes and Basidiomycetes, respectively.

a. The ascocarp. The ascocarp consists of an outer sterile sheath of compacted hyphae derived from the vegetative mycelium and an inner fertile portion comprising at first the sexual apparatus and later the product of sexual interaction. This latter consists of a fascicle of more or less extensively branched hyphae, the ascogenous hyphae, which, originating in the "germination" of the fertilized female gametangium, contain conjugately dividing nuclei of both sexual mates. The terminal cells of the ascogenous hyphae each receives a pair of nuclei, one of either mate, and becomes differentiated as an ascus, within which nuclear fusion and meiosis occur in rapid succession. Meiosis is typically followed by one mitotic division in the ascus, and the eight haploid nuclei serve as organizing centers for the free-cell formation of eight ascospores. (There are relatively few exceptions: four binucleate spores, or larger numbers of spores as the result of additional mitotic division.) The ascospores of many species are arranged in uniseriate order and accurately reflect the basic events of meiosis (see *Tetrad Analysis*, Section V, A, 1). In all ascocarps, the system of ascogenous hyphae is generally acknowledged to be a dikaryotic phase (Olive, 1953; to the contrary, Martens, 1946) and is transient, restricted in its development, parasitic upon the vegetative mycelium, and incapable of independent existence and growth. The details of the development of the ascocarp vary considerably. In some forms, e.g., *Ascobolus*, initiation of the ascocarp is delayed until the induction of the female sexual organ by the presence of a compatible male inducing and fertilizing element, and the entire developmental sequence is coordinated with that of the sexual elements within (Bistis, 1956b, 1957). More commonly, however, e.g., *Neurospora*, the female sexual organ, contained within an ascocarp primordium, the protoperithecium, forms without male stimulation, and

further development awaits fertilization (Ames, 1934; Backus, 1939; Zickler, 1952).

On the basis of their morphologies at maturity, three types of ascocarps are recognized.

Cleistothecia are closed ascocarps each containing from one to many asci; these may form a columnar layer, e.g., "downy mildews," or may be randomly distributed throughout the interior of the fruiting structure, e.g., *Aspergillus* and *Penicillium*. Neither the cleistothecium nor the individual ascus is provided with means of dehiscence, and spore dispersal depends upon the disintegration of the fruiting structure.

Perithecia differ from the former in that the ascocarp is usually flask-shaped but in all cases is provided with an opening through which the ascospores are discharged, e.g., *Neurospora, Sordaria*, and *Podospora*. A fascicle of asci numbering from ten to a hundred or more is borne in the base of the perithecium. At maturity, each ascus in succession elongates to bring its tip into the opening of the perithecial neck; the tip of the ascus ruptures, the spores are forcibly discharged into the air, and the emptied ascus collapses.

Apothecia differ from perithecia in the lesser extent of the sterile, outer sheath, which is not extended upward to form a covering over the developing asci, e.g., *Ascobolus* and *Morchella*. The apothecium is thus an open cup and at maturity exposes the hymenium, a continuous, columnar layer of asci interspersed with sterile hairs. Apothecia vary in size from barely visible to several inches in breadth. The hymenium is usually flat, but it may be folded and wrinkled in some of the larger forms, such as the morels.

b. The basidiocarp. In the Basidiomycetes, exclusive of the rusts and smuts, the basidiocarp is an imposing structure recognized by the casual observer as a "jelly fungus," a "bracket fungus," a "mushroom," a "puffball," or a "stinkhorn." In these forms, the entire basidiocarp is roughly homologous to the ascogenous hyphal system of the Ascomycetes, since the entire structure is typically composed of dikaryotic hyphae, i.e., the product of sexual interaction. Following nuclear exchange in dikaryosis (see below) and the establishment of the dikaryotic, or secondary, mycelium—which, unlike the dikaryophase of the Ascomycetes, is capable of independent and indefinite growth—the basidiocarp originates, at least in higher forms, in the extensive proliferation of a single cell (Buller, 1931). Although most basidiocarps are produced upon and are composed of dikaryotic hyphal elements, fruiting bodies may in certain cases be formed upon heterokaryotic mycelia of other types or upon homokaryotic

mycelia (Swiezynski and Day, 1960b; Raper and Krongelb, 1958; Parag and Raper, 1960). Initial hyphal proliferation produces a loose clump of mycelium, which with further growth becomes more compact. The pattern of further development varies from group to group. In all cases, however, extended hymenial surfaces are eventually formed. The hymenium consists of a columnar layer of basidia, which are borne terminally upon subtending hyphae and within which occur nuclear fusion and meiosis. Following meiosis, the daughter nuclei migrate into lateral or terminal outgrowths of the basidium, and at the tips of these, basidiospores are formed. Basidia, at maturity, may be either septate or unicellular, and the number of basidiospores per basidium may be two, most commonly four, and sometimes eight or more. The major types of basidiocarps differ in the degree of structural differentiation and particularly in the means by which the extensive hymenium is accommodated, as, in all but the simpler forms, the area of the hymenium is many times greater than the total surface area of the fruiting body.

In the simple arachnoid and "jelly" fungi, relatively little specialization occurs, the mature fruiting structure consisting of intertwined hyphal elements and bearing a hymenium on the exposed surface. In more advanced forms, a greater degree of specialization occurs, with the achievement of large and elaborate structures. Here, two developmental patterns may be recognized: indeterminate and determinate. (1) In indeterminate forms, the basidiocarp may become functionally mature, with an active spore-bearing hymenium, while still a relatively simple structure. Growth continues, however, with the enlargement of the structure and the continued expansion of the hymenium, which may become disposed in pores, upon spines, or upon platelike ridges, gills. The gilled form *Schizophyllum,* which initiates sporulation while a tiny, shallow, hymenium-lined cup and proceeds to the elaboration of a sizable fruiting structure with radiate, split gills, typifies indeterminate development. Many of the indeterminate forms are long-lived and continue to shed spores during an entire season; some are perennial, with renewed growth each season for several years. (2) In determinate forms, by contrast, the pattern of development is much more complex and is well illustrated by the common mushroom. Over a period of time, there is a slow accumulation of mycelium and food materials in a definitive body, the "button," which is often formed just under the surface of the soil. Growth of the "button" is accompanied by the internal differentiation of the mycelial elements into all the structural features of the mature fruiting body. When the "button" is mature, it absorbs great quantities of water, certain tissues elongate enormously while others swell, and there

result the emergence and unfolding of the fully preformed structure—the legendary "growth" of mushrooms (Bonner, 1956). Mushrooms are usually transient; their spores are formed and discharged within a short period of time, and the fruit bodies then decompose. The "puffballs" and related forms, in which the hymenium lines the walls of small internal cavities, are also determinate; there is a period of growth prior to functional maturity, at which time all the spores are produced. The spores are subsequently released by the disintegration of the fruiting body.

The foregoing description of the vegetative and reproductive features of the fungi is intended only as a broad, generalized survey of fungal morphology; a really comprehensive treatment would cite exceptions by the score. In this brief account, however, we hope to have conveyed something of the "flavor"—the frequent occurrence of novel features and the wide range of variability—of the fungi.

C. Cytology

Historically, the cytology of the fungi has lagged somewhat behind that of other major groups of plants and animals. The minute size of the cellular components of the fungi has been primarily responsible for this retarded growth and has had two notable consequences: (1) with the techniques available at any time, observations were often marginal, and conclusions frequently incorporated a rather high content of interpretation; (2) widely ranging interpretations of structures and events seen near the limits of visibility have generated numerous controversies, the solutions of which have, in cases, had to await for decades the introduction of resolving techniques. The half-century debate over "double fertilization and brachymeiosis" in the Ascomycetes perhaps best illustrates this aspect of the progress of fungal cytology. Well before the end of the last century, the association of male and female nuclei in the process of gametangial fusion was established for a number of representative species. According to some observers, nuclear association was immediately followed by nuclear fusion; according to others, the nuclei became associated, but no fusion occurred at this time. Everyone agreed, however, that nuclear fusion did occur at a later stage, in the developing ascus. The primary (fused) nucleus of the ascus was thus tetraploid and diploid according to the two schools of thought, and the third nuclear division in the ascus was thus a third meiotic division, i.e., brachymeiosis, or mitosis. An increasing awareness of the true nature of the dikaryophase together with the accumulation of voluminous genetic evidence slowly resolved the matter to the satisfaction of all—perhaps. With the present

accelerated introduction of techniques and methodologies, controversies tend to be of less duration if no less numerous.

Beginning in the later part of the last century, the extensive work of Claussen, Guilliermond, Dangeard, Harper, and others well delineated the basic cytological features of the fungi. The extension of their work, both in scope and in detail, has strongly emphasized the universality of cellular organization. Cytologically, the fungi have been found to differ from other organisms only in minor details, and the significance of present cytological work on fungi appears to lie in the exploitation of small differences rather than the clarification of common features. The older cytological work is well covered in the standard texts of Gwynne-Vaughan and Barnes (1937) and Gäumann-Dodge (1928), while more recent reviews of Cutter (1951) and Olive (1953) bring the subject to essentially its present status with certain exceptions noted below.

1. Cytoplasm

As viewed with the light microscope, either in living or stained condition, the cytoplasm of fungi differs in no consistent way from that of other organisms: a finely granular ground material in which are suspended vacuoles, fat droplets, and a variety of larger granules and particles. Certain groups, as elsewhere, have their characteristic cytoplasmic inclusions: the volutin granules of the yeasts, the nuclear cap of the motile cells of the uniflagellated Phycomycetes, the several categories of locomotor apparatuses in the motile cells of the aquatic fungi, etc.

During the past few years, an increasing number of studies of fungal materials with the electron microscope have appeared. Here again, the fine structure of fungi conforms to that of other materials, and only minor features peculiar to the fungi have been noted. Thus at the submicroscopic level, fungal flagella repeat the 9 + 2 pattern of animal cilia, algae flagella, etc.; mitochondria strongly resemble those of other organisms, although they may in general be somewhat less elaborate in detail; the nuclear membrane, constituted of two layers and interrupted at intervals by pores, has the same organization as that of higher plants and animals; and an interconnecting system of tubules and vesicles bounded by membranes resembles, or indeed is identifiable as, an endoplasmic reticulum.

The range of fungal materials that has been examined with the electron microscope is still quite small, however, relative to the sum of the literature dealing with submicroscopic structure. Studies of fine structure of phycomycetous material is primarily restricted to the locomotor apparatus (Manton *et al.*, 1951, 1952; Koch, 1956) and nuclear cap

(Blondel and Turian, 1960) but includes at least one figure of a vegetative cell of a terrestrial form, *Syncephalastrum* (Moore and McAlear, 1961a). The Ascomycetes are better represented, with studies of yeasts and related forms by O'Hern and Henry (1956), Agar and Douglas (1957), Hashimoto *et al.* (1958a, b), Conti and Naylor (1959), and Edwards *et al.* (1959); of the filamentous form *Neurospora* by Tsuda (1958), Shatkin (1959), and Shatkin and Tatum (1959); and of the imperfect form *Stilbum* by Moore and McAlear (1962). Only a few Basidiomycetes have been examined; a rust, *Uromyces*, by Moore and McAlear (1961b) and a few hymenomycetous forms, including *Coprinus* by Heitz (1959), *Polystictus* by Girbardt (1961a), and *Schizophyllum*, etc., by Moore and McAlear (1961a, 1962).

Two recently described structures serve to illustrate the sort of novel details that have been revealed to date in the fungi through electron microscopy.

In vegetative hyphae of each of a number of fungi—one Phycomycete, three Ascomycetes, and five Basidiomycetes—Moore and McAlear (1961a) report an amorphous, spongelike body, the lomasome, in the outer layer of protoplasm but external to the plasma membrane and contiguous with the cell wall. Lomasomes were found in vegetative cells, but not in spores or other specialized cells. The authors suggest that this body, unreported in cells of other materials, may originate in the outward migration of cytoplasmic vesicles and the incorporation of their membranes into the plasma membrane.

The septa of several species of Basidiomycetes have also recently been shown by Moore and McAlear (1962) and Girbardt (1961a) to have a novel structure. The inner perimeter of the pore is broadly flanged and is covered on either side by a dome-shaped, enclosing fold of the endoplasmic reticulum. The subject of the observations of Girbardt was the dikaryotic mycelium of *Polystictus*; the mycelia of the several species with which Moore and McAlear worked were derived from basidiocarps and were presumably also dikaryotic. In view of the apparent lack of nuclear migration in the dikaryotic mycelia of at least two species, *Coprinus lagopus* (Swiezynski and Day, 1960a) and *Schizophyllum commune* (P. S. Snider, personal communication), the homokaryotic mycelia, through which nuclei migrate with considerable celerity (Buller, 1931; Snider and Raper, 1958), might have been expected to have septal pores that are larger in diameter and simpler in structure, comparable to those pictured for the Ascomycetes *Neurospora* (Shatkin and Tatum, 1959), *Mollisia*, and *Aspergillus* (Moore and McAlear, 1962). In a recent paper, however, Snider (1963) states, "Current electron micro-

seopie comparisons of the structure of septal pores in homokaryotic and dikaryotie hyphae of *Schizophyllum* have indieated to date no differenee in the size or the strueture of these pores (Snider, unpublished)." This observation—its confirmation assumed—makes even more perplexing the mechanies of nuclear migration in homokaryotic hyphae (Buller, 1931; Raper and San Antonio, 1954; Snider and Raper, 1958; Swiezynski and Day 1960a). The evidence from these studies has strongly indicated the migration of whole nuclei, and of this the reeent report of Snider (1963), which gives definitive evidenee of the transport of complete genomes *through* the hypha, leaves little doubt. Snider's query of how small a pore (ea. 50 mμ in the eases figured to date) the nuelei of fungi (ca. 2–3 μ) ean pass through is sure eventually to evoke some interesting answers. Even the passage of a nueleus through the larger and simpler pore of *Neurospora*, pietured by Shatkin and Tatum (1959), requires a very tight squeeze!

2. Nucleus

It has long been the eonsensus of fungal eytologists that the fungal nueleus, though small (one to a few microns), has the same basic structure as the nuelei of higher plants and animals: a single nucleolus and a network of ehromatin strands enclosed within a nuclear membrane. In most forms, the resting, vegetative nuclei are very small, but they enlarge somewhat prior to division. The diploid nuclei just prior to meiosis, however, are mueh larger, and a major portion of the literature on nuelear strueture and division is eoneerned with the meiotic phase of the life eyele. Nuelear division in all cases appears to be internuelear, the nuelear membrane persisting throughout (Olive, 1953).

a. Meiosis. The classieal eytologieal work of Harper and others about the turn of the eentury demonstrated meiosis in numerous groups of fungi to follow the same sequence as in other organisms. Even in meiosis, however, the ehromosomes and other elements of the division figures are exceedingly small, and the stains and proeedures available did not permit adequate resolution of chromosomes and detailed features of the spindle. As a result, chromosome counts and other details were often in dispute. New impetus was given fungal eytology in 1945 by MeClintoek, who, in a study of *Neurospora crassa*, first used the aceto-oreein teehnique with fungal material and was able to clarify many previously obseure details of the meiotic process. McClintock again emphasized the similarity of the meiotie process in this fungus to that in higher forms. Later eytologieal investigations of *Neurospora* by Dodge *et al.* (1950) and Singleton (1953) confirmed and extended McClintock's

findings. Singleton (1953) cites synapsis of extremely contracted chromosomes, which elongate only after synapsis, as the only cytological feature of *Neurospora* that makes it different from other organisms (including other fungi?). The application of the new techniques, which also include Giemsa and Feulgen staining, has not only enhanced the study of cytology per se, but has also made chromosome counts a most useful adjunct of experimental work. The latter is exemplified in the study of polyploidy and hybridization in the uniflagellated water mold *Allomyces* (Wilson, 1952; Emerson and Wilson, 1954) and in the Aseomycete *Podospora* (Franke, 1962), and in the reexamination of the life cycle of the biflagellated form *Pythium debaryanum* (Sansome, 1961).

b. Mitosis. The investigation of the more difficult matter of the division of vegetative nuclei has also aeeelerated during the past decade. In an evaluation of the cytologieal evidence prior to 1953, Olive concluded that division of vegetative nuelei of fungi is mitotic. This conclusion has since been both challenged and supported by numerous recent studies of nuclear division in a wide range of vegetative mycelia.

Robinow (1956) found insuffieient evidenee for mitosis and suggested that, although meiosis in fungi had been shown to be typical, the division of vegetative nuclei might well be of a less highly evolved type resembling the division of the nuclear bodies of the bacteria. A number of detailed investigations of numerous fungi representing all major groups have failed to reveal the typical structures of mitosis; they have rather shown an amitotic process consisting of the condensation of the chromatin material, the degeneration of the nueleolus, and the pulling apart of the chromatin material as two teardrop daughters. These studies included as materials *Mucor hienalis* and *M. fragilis* (Robinow, 1957a), *Phycomyces blakesleeauus* (Robinow, 1957b), *Endogne sphagnophila* (Bakerspigel, 1958), *Blastocladiella emersonii* (Turian and Cantino, 1960), several biflagellate water molds (Bakerspigel, 1960), *Blastomyces dermatitidis* (Bakerspigel, 1957), *Neurospora crassa* (Bakerspigel, 1959a; Weijer and Dowding, 1960), *Phyllosticta* sp. (Bakerspigel, 1959b), *Schizophyllum commune* (Bakerspigel, 1959c), and *Polystictus versicolor* (Girbardt, 1960, 1961b). In the yeast *Lipomyces lipofer*, Robinow (1961) has recently described chromosome-like bodies whieh appear to divide transversely. These centrally constricted bodies then become arranged in a "stack" from whieh adjacent halves eondense and move as two teardrop daughters to opposite poles.

In direct contradiction to the findings cited above, other investigators have reported the essential features of mitosis in a number of representative forms. Not only is one speeies, *Neurospora crassa*, common to

the two groups, but all investigators have employed the same staining techniques. Among the forms in which vegetative nuclear division is reported to be mitotic are *Hypomyces solani* f. *cucurbitae* (El-Ani, 1956), *N. crassa* (Somers *et al.*, 1960; Ward and Ciurysek, 1962), *Puccinia graminis* (McGinnis, 1953), and an unidentified Basidiomycete (Ward and Ciurysek, 1961).

Aside from *N. crassa,* which obviously requires definitive clarification, the possibility of course remains that there exist among the fungi two or more basic modes of division of vegetative nuclei. Neither the history of fungal cytology nor, more importantly, the distribution of forms studied to date, however, gives much promise of resolving the controversy in such a mutually satisfactory manner.

The over-all cytological picture of the fungi may be summed up in the conclusion that the fungal cell differs from most other cells in one important respect: it is smaller.

III. Life Cycles and Sexuality

Something of the variability and diversification of the fungi has been shown above in reference to vegetative and reproductive morphology. These features superficially reflect in part the more basic diversifications of life cycles and sexual phenomena, in which realm the fungi achieve their most bizarre and novel distinctions as compared to other groups of plants and animals. From the earliest days of experimental mycology, sexuality and associated phenomena have occupied a position of central interest, and perhaps no other aspect of fungal studies has presented the investigator with so many puzzling contradictions.

The experimental study of sexuality approaches its centennial anniversary. Through the work of De Bary, Brefeld, Pringsheim, van Thieghem and others, the life cycles and the sexual apparatuses of many broadly representative species of fungi were well known by the end of the last century. The discovery of heterothallism in the "black bread molds" by Blakeslee in 1904 provided great stimulus to the broader study of sexuality in fungi, which during the last half century has yielded a voluminous literature.

The subject of fungal sexuality has become rather highly specialized, and numerous treatments of the field have appeared as it has developed. Among the earlier comprehensive reviews, *Die Sexualität der niederen Pflanzen* of Kniep (1928) is preeminent, both in scope and in perceptiveness. This and other contemporary works devoted wholly or in part to the subject by Gäumann (1926), Gäumann-Dodge (1928), Link (1929), and Hartmann (1943) integrated the information regarding

fungal sexuality into the broader problem of sexuality as a basic biological phenomenon. The field has been increasingly active during the past two decades, but unfortunately, no comprehensive treatments have appeared during this time. A number of reviews by Whitehouse (1949a,b, 1951), Burnett (1956), Olive (1958), Raper (1953, 1954, 1959, 1960), and Esser (1963, in press) have treated limited aspects of the problem. Only an outline of the facts relating to and a brief consideration of the significance of fungal sexuality can be included in the present account (for fuller detail and a more complete bibliography, cf. Raper, 1954, 1960).

Plasmogamy, karyogamy, and meiosis, the irreducible components of sexuality, impose a cyclic progression of sequential stages upon all organisms with sexual reproduction. Alteration of the basic cyclic progression may be brought about by three major features: (1) vegetative or somatic growth is intercalated into the cycle at different points and thereby determines the basic types of life cycles; (2) innate restrictions upon universal compatibility predestine a variety of patterns of sexuality; and (3) morphological and physiological differentiation results in different sexual mechanisms, the mechanical means for the juxtaposition of compatible sexual elements, and in different developmental histories. The fungi are rich in variants of each of these three aspects and, in the numerous combinations of variants, provide a range of diversification probably unmatched in any other group of organisms.

A. *Life Cycles*

The general impression among biologists that the fungi are haploid organisms is perhaps the most nearly accurate simple generalization that is possible, but it fails to recognize (1) essential phases that occur in the developmental histories of about half the fungi and (2) numerous forms that lack a protracted haploid phase. In addition to the haploid and diploid nuclear phases, a third nuclear phase, the dikaryophase, is interposed between plasmogamy and karyogamy in the Euascomycetes and Basidiomycetes. The dikaryophase is peculiar to the fungi. The dikaryon originates following plasmogamy in the intracellular association of pairs of compatible nuclei, which are of identical or different origins in self-fertile and cross-fertile species, respectively. The associated pairs of nuclei, during subsequent vegetative growth, divide synchronously, i.e., by conjugate division, to establish and maintain between the two nuclear types a ratio of unity. The dikaryon is thus a highly specialized type of heterokaryon (see below). The dikaryophase occurs as the ascogenous hyphal system of the Euascomycetes and as the

secondary mycelium of the Basidiomycetes and has thus become, as Whitehouse (1951) has emphasized, a major component of the sexual history in these forms. That the dikaryon exists only in the two higher groups of the fungi indicates their evolutionary relationship. The absence of the dikaryophase in the simpler Ascomycetes and the further fact that the dikaryon of the Ascomycetes is parasitic upon the vegetative mycelium and is restricted in growth, while that of the Basidiomycetes is independent and capable of indefinite growth, strongly suggest the main course of evolution of the dikaryon. In both groups, the dikaryophase leads to and is terminated by the events of karyogamy and meiosis in homologous specialized cells, the asci and basidia.

1. Life Cycles of Fungi

Seven different sequences of nuclear phases in respect to vegetative growth and the events of plasmogamy, karyogamy, and meiosis characterize the life cycles that occur in the fungi. These life cycles are diagrammatically represented in Fig. 3.

a. Asexual cycle. A very sizable fraction of all fungal species (ca. 20% according to Bessey, 1950) have no known sexuality. Strictly speaking, these constitute the large class, Fungi Imperfecti, but many species may be assigned to the other classes on the basis of nonsexual features, and most imperfects appear to be allied to the Ascomycetes. Such forms,

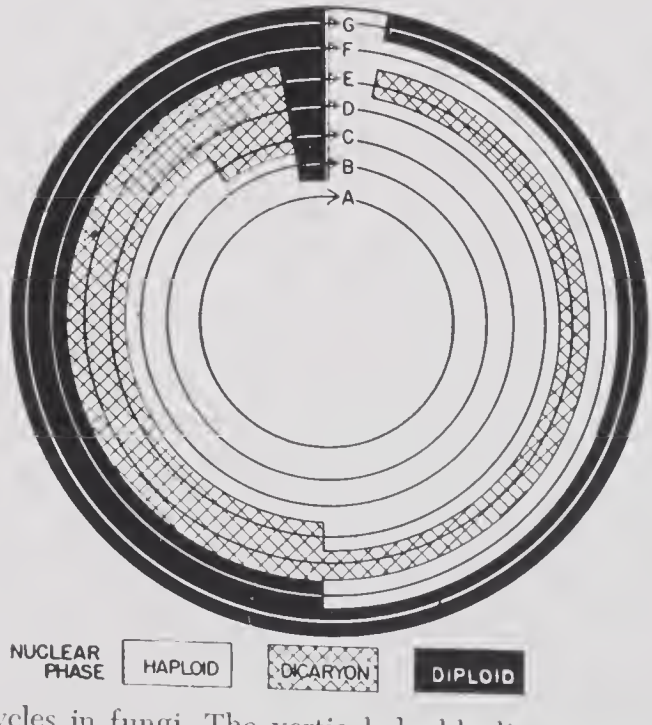

FIG. 3. Life cycles in fungi. The vertical double line at top represents meiosis; the two narrow, adjacent sectors each represent one nuclear germination. From Raper (1954).

which reproduce only by vegetative or asexual means, pass through no change in nuclear phase and may thus be considered as haploid. In many imperfect species, however, the otherwise possibly dire consequences of the lack of sexuality may be effectively mitigated by heterokaryosis and parasexuality, the latter involving sporadic and infrequent diploidy (see below).

b. Haploid cycle. In a majority of the Phycomycetes and the Protoascomycetes, the vegetative stage of the life cycle is haploid, and the diploid phase is confined to a single nuclear generation, the fusion or zygote nucleus.

c. Haploid-restricted-dikaryotic cycle. In the higher Ascomycetes and coincident with the production of complex ascocarps, a dikaryophase, i.e., the ascogenous hyphal system, precedes karyogamy. The vegetative mycelium is exclusively haploid and serves as host for the dikaryophase. The dikaryophase in the Euascomycetes varies from a short chain of cells in certain of the "downy mildews" to a very extensive hyphal system producing a hymenium of great extent in the morels.

d. Haploid-dikaryotic cycle. The typical life cycle in the Basidiomycetes alternates between two independent vegetative phases, haploid and dikaryotic. The termination of the haploid and the initiation of the dikaryotic phase, however, depends, in obligatorily cross-mating forms, upon the chance juxtaposition of compatible haploid mycelia. Both mycelial mates become reciprocally converted into dikaryotic mycelia, and the eventual basidiocarps produced thereon are composed exclusively of dikaryotic elements.

e. Dikaryotic cycle. An operational variant of the preceding cycle occurs in some smut fungi, in which the dikaryophase is initiated by the fusion of sporidia (basidiospores). The entire vegetative phase is thus dikaryotic, and the haploid and diploid phases are each limited to one nuclear generation.

f. Haploid-diploid cycle. An alternation of extended haploid and diploid vegetative phases is known to occur only in the Blastocladiales of the uniflagellate series of Phycomycetes, e.g., *Allomyces*, in which the gametophytic and sporophytic generations are distinguishable only by their reproductive elements.

g. Diploid cycle. The diploid cycle, characteristic of the animal kingdom, occurs in fungi only in the yeasts and a few members of the Blastocladiales. In the former, the haploid phase may be extended beyond a single cellular generation if mating is not achieved between the primary meiotic products, the ascospores; in the latter, a single mitotic division is interposed between meiosis and syngamy.

B. Patterns of Sexuality

Blakeslee, in 1904, discovered the first case among fungi of obligatory cross-mating in the "black bread mold" *Rhizopus nigricans*; during the next few years, he and others found cross-mating to be very common among the Mucorales and established that cross-mating was determined by two alleles at a single locus (Blakeslee, 1906; Burgeff, 1912, 1914). The requirement for sexual interaction between two self-sterile strains Blakeslee termed *heterothallism*; the antithetical condition, sexual interaction between elements of a single, self-fertile strain, was termed *homothallism*. In the intervening years, obligatory cross-mating has been demonstrated in a large number of fungi, representative of practically all groups, and the genetic devices determining the pattern of intermating have been shown to be quite diverse. Homothallism has likewise been shown to be equally widely distributed.

Blakeslee's original conclusion of sexual differentiation between (+) and (—) strains has never been satisfactorily substantiated; of numerous subsequently described cases of heterothallism, some few instances clearly involve sexual differentiation, whereas most equally clearly do not. It has long since been recognized that sexual dimorphism is a less common basis for obligatory cross-mating among fungi than is incompatibility, which in certain cases does not restrict to two the number of self-sterile, cross-fertile classes. The simple distinction between homothallism and heterothallism has also become somewhat blurred as new situations have been described. Numerous findings have contributed to this loss of distinction; of these, the more important follow. (1) The concept of the individual has undergone change, and with the change have come different interpretations of differentiation within the clone. This applies particularly to holocarpic forms, in which each "individual" is differentiated in its entirety as a sexual element. (2) A self-fertile mycelium may contain two genetically different, self-sterile and cross-fertile nuclear components (see *Secondary homothallism* below). (3) Self-sterile, cross-fertile strains have been derived via mutation from self-fertile, wild strains in numerous species. Certain authors (Wheeler, 1954) term such strains heterothallic; other authors retain homo- and heterothallism to designate the natural status of the species.

It has been pointed out earlier (Raper, 1960) that control of sexual processes is imposed at two different levels: (1) the basic genetic predestination of future sexual capacities and (2) the genetic-metabolic apparatus required for normal sexual development. The pattern of sexuality is imposed as a primary genetic control and is fixed at meiosis

by the segregation of (*a*) neither sexual nor incompatibility factors, (*b*) sexual factors, or (*c*) incompatibility factors. The first of these determines universal compatibility, homothallism; the latter two determine two types of heterothallism.

1. Homothallism

Perhaps one of the more important facts about patterns of sexuality among the fungi is the prevalence of homothallism. It is characteristic of perhaps a majority of all fungal species and occurs in all major groups. Self-fertile species, however, may be subdivided into two categories: (*a*) true or primary homothallism, those cases in which there is no differentiation among individual nuclei in respect to sexual or mating competence; and (*b*) secondary homothallism, those cases in which such differentiation is present but does not ordinarily prevent self-fertility.

a. (Primary) Homothallism. Primary homothallism is epitomized by the ability of identical nuclei to fuse in a sexual interaction and must assume a physiological, intramycelial differentiation, which may be accompanied by the differentiation of male and female sexual organs, e.g., Phycomycetes and Ascomycetes, or may occur between individual nuclei in the absence of sexual organs, e.g., Basidiomycetes. Two different and opposed aspects of the biological role of homothallism probably account for its prevalence. (1) Sexual reproduction, even when it involves no possibility of genetic recombination, provides as end products of the reproductive process either highly resistant spores, in most Phycomycetes and Ascomycetes, or the only type of spore adapted to wide dispersal, in most Basidiomycetes. (2) Even though a very high percentage of homothallic sexual reactions bring together like genomes, occasional interactions between sexual elements of different individuals of diverse origins—observed in numerous forms and mechanically possible, hence probable, in most or all species—would provide sufficient interplay of genetic materials to maintain the genetic variability necessary for adaptation. Intermatings of different homothallic strains serve as the basis for a number of recent significant genetic studies (see below).

Real or apparent changes in the basic fact of homothallism have been achieved in a number of forms by two different types of alterations in the genetic materials. (1) In *Allomyces*, hybridization between two homothallic species—each comprising a polyploid series and having 16 and 28 chromosomes, respectively, in the strains employed—yielded a wide range of aneuploid F_1 progeny, some of which were predominantly of

one sexual sign, either ♂ or ♀ (Emerson and Wilson, 1954). Selection in subsequent generations produced practically pure (ca. 99%) unisexual lines (Machlis, 1958a). (2) In a number of homothallic Ascomycetes, point mutations result in self-sterility but permit cross-fertility. Details of this work and its relevance to patterns of sexuality other than homothallism will be considered below.

b. Secondary homothallism. Homothallism, as considered above, reflects the inherent competence of homokaryotic strains (and, potentially, of individual nuclei) for self-fertility. The mycelium may in certain cases be self-fertile by virtue of the presence of two self-sterile but compatible nuclear components. Stable heterokaryons of this type characterize numerous four-spored species of Ascomycetes, e.g., *Neurospora tetrasperma* (Dodge, 1928b), *Gelasinospora tetrasperma* (Dowding, 1933), *Podospora anserina* (Ames, 1934), etc., and a few two-spored Basidiomycetes, e.g., *Aleurodiscus canadensis*, *Conocybe tenera* f. *bispora*, etc. (Sass, 1929; Skolko, 1944). The heterokaryotic association is established at spore formation with the inclusion of two meiotic products, and the superficial result is homothallism ("secondary homothallism," Whitehouse, 1949a; "pseudohomothallism," Dodge, 1957), but the basic pattern of sexuality is like that of related heterothallic species. The means by which compatible nuclei are paired in the developing ascus differs in *Neurospora tetrasperma* (Dodge *et al.*, 1950) and in *Podospora anserina* (Rizet and Engelmann, 1949; Franke, 1957).

2. *Heterothallism*

Species characterized by obligatory cross-mating are interspersed among homothallic species throughout the fungi. A number of different genetic devices determine both the fact and the pattern of cross-mating. There are two major types of differentiation in respect to mating competence, sexual dimorphism and incompatibility, respectively responsible for "morphological heterothallism" and "physiological heterothallism" (Whitehouse, 1949a).

a. Sexual dimorphism. The differentiation of individuals as male and female as the basis for cross-mating is relatively uncommon among the fungi, and even where it does occur, there is frequent uncertainty of the cause of differentiation. Thus, among the more primitive, holocarpic Phycomycetes, "heterothallism" is a mechanical necessity, but whether it is genetically or physiologically determined has not been established in any case. The heterothallic members of the biflagellate Phycomycetes, e.g., *Achlya* spp. and *Dictyuchus monosporous*, show marked sexual dimorphism, but the underlying pattern of sexuality is

complex, and the genetics of the system remains to be investigated (Couch, 1926; Raper, 1940 *et seq.*; Barksdale, 1960). Among the Ascomycetes, sexual dimorphism is known with certainty in two cases, *Ascosphaera* (*Pericystis*) *apis* (Spiltoir, 1955) and in certain species of the Laboulbeniales (Thaxter, 1896; Benjamin and Shanor, 1950), a group of highly specialized parasites of insects. Again, the underlying basis of male-female differentiation is not known. Additional reports of sexual dimorphism in the Ascomycetes *Hypomyces* (Hansen and Snyder, 1946) and *Stromatinia* (Drayton and Groves, 1952) and in the Basidiomycetes *Solenia* (Greis, 1942) occur in groups characterized by incompatibility, and it seems probable that in these, dimorphism reflects secondary defects (probably mutative) rather than basic patterns of differentiation (Raper, 1959).

b. Incompatibility. Of far more common occurrence and far better understood is the restriction of mating competence by incompatibility factors, extrasexual genetic determinants that underlie the differentiation of two or more morphologically identical classes that are typically rigidly self-sterile and cross-fertile. Mating between individuals of compatible classes is usually reciprocal. Heterothallism due to incompatibility is the probable case in the most advanced Phycomyces, the Mucorales, and it is characteristic of the heterothallic members of the Ascomycetes and Basidiomycetes. Mating systems based on incompatibility vary considerably in detail, and the following briefly relates the different types of incompatibility with the major groups of these two classes of fungi.

Heterothallism in the yeasts, first demonstrated by Winge and Laustsen (1939a, b), involves two alleles, *a* and *α*, at a single locus (Lindegren and Lindegren, 1943). The mating-type factors, however, appear to be unstable, with sufficiently frequent mutation to alternate, compatible alleles to permit a low degree of self-fertility (Lindegren and Lindegren, 1943, 1944; Leupold, 1950; Ahmad, 1952, 1953), and a compound incompatibility locus with multiple alleles has been reported in one species (Leupold, 1958a, b). The operation of incompatibility may also further be affected by additional factors that result in self-fertility (Winge and Roberts, 1949; Takahashi, 1958); and the simple expression and segregation of incompatibility factors may be considerably confused by polyploidy (Lindegren and Lindegren, 1951; Roman *et al.*, 1951, 1955; Subramaniam, 1951; Pomper *et al.*, 1954; Leupold, 1956).

Among the filamentous Ascomycetes and the rusts of the Basidiomycetes, heterothallism is due to the segregation of two alternate alleles, *A* and *a*, that prevent self-fertility and allow reciprocal mating

between functionally hermaphroditic individuals of two classes, individuals of each class producing differentiated male and female sexual organs (Lindegren, 1932, 1936; Ames, 1934; Dodge, 1932, 1935; Backus, 1939; Langford and Keitt, 1942; Keitt *et al.*, 1946; Catcheside, 1951; Bistis, 1956b, 1957). The incompatibility alleles here are apparently very stable, as no report of the mutation of the factors appears in the voluminous literature for *Neurospora*, *Ascobolus*, *Venturia*, etc.; neither is there any published evidence of a compound incompatibility locus. Sexual interaction between individuals of compatible classes is reciprocal, but recent work with *Ascobolus* has illustrated minor differences in reciprocal crosses, the most significant difference being the nonreciprocal initiation of the sexual progression (Bistis and Raper, 1963).

Incompatibility in the Basidiomycetes exclusive of the rusts and the smuts differs in three conspicuous ways from that just considered: (1) in many cases there are two series of incompatibility factors, *A* and *B*, rather than one; (2) there is invariably an extended series of alternate *A* or *A* and *B* factors; and (3) there are no differentiated sexual organs, and plasmogamy is always by fusion of vegetative cells. One series or two series of incompatibility factors determine bipolar and tetrapolar incompatibility, respectively. In the former, the progeny of a single fruit are equally divided between two self-sterile classes, whereas in the latter, four classes are produced in equal frequency. In either case, fertile mating occurs only between strains that have no incompatibility factor in common. The more distinctive feature of this type of incompatibility, however, is the very large number of distinct mating types in the natural population: in bipolar species, the number of mating types equals the number of alternate factors of the single series, while in tetrapolar species, the number of mating types equals the product of the numbers of alternate factors in the two series. Inbreeding in the two cases is restricted to 50% and 25%, respectively, but outbreeding is practically unrestricted, $> 90\%$ in either case with 10 or more alternate factors (Mather, 1942; Whitehouse, 1949b). The genetics of tetrapolar incompatibility will be given in some detail in a later section.

C. The Sexual Progression

Beyond the variations in life cycles and basic patterns of sexuality, additional variety is provided by the specific means by which compatible sexual elements are brought together and by variations in the course of the sexual progression before and after "fertilization."

1. *Sexual Mechanisms*

Kniep (1928) recognized in plants and animals four basic types of sexual mechanisms, all of which are of common occurrence among the fungi.

a. Gametic copulation, the fusion of differentiated, uninucleate gametes, is typical of many members of the uniflagellate Phycomycetes, e.g., *Allomyces*. In some cases, the planogametes are morphologically differentiated as male and female; in others, they are indistinguishable.

b. Gamete-gametangial copulation, the fusion of a differentiated, uninucleate gamete of one sexual sign with a larger gametangium, that produces no differentiated gametes of the other sign, occurs in the biflagellate Phycomycetes, e.g., *Achlya* (differentiated ♀ gamete), and in certain of the Ascomycetes, e.g., *Neurospora* (differentiated ♂ gamete).

c. Gametangial copulation, the fusion of two specialized sexual cells, neither of which differentiates gametes, is found in the Mucorales of the Phycomycetes and in many Ascomycetes, e.g., *Pyronema*. In the former case, the two interacting gametangia are morphologically alike in heterothallic species and unlike in most homothallic species; in the Ascomycetes, they are usually clearly distinguishable as ♂ and ♀.

d. Somatic copulation, the fusion of undifferentiated vegetative cells, marks plasmogamy in the haploid-diploid yeasts and in the initiation of the dikaryophase in most Basidiomycetes. In the latter, nuclear exchange is reciprocal. Depending upon interpretation, two additional cases may be included here that involve the fusion of the immediate products of meiosis without differentiation as gametes: ascospores of certain yeasts and sporidia (basidiospores) of numerous smuts.

2. *The Developmental Sequence*

Through the interplay of the three facets of sexuality outlined above, life cycle, pattern of sexuality, and sexual mechanism, the fungi embrace a wide array of developmental histories. Such correlations as appear to exist between these histories and the major phylogenetic groupings have been examined previously (Raper, 1954). More relevant to our present purpose is a brief consideration of the means by which intrinsic controls of sexual phenomena find their expression in the developmental sequence.

Little is known of the means by which primary control is exerted. In the case of incompatibility, the block to intraclass mating is different in two Ascomycetes from which definitive, if fragmentary, information

is available, and both of these are quite different from that in a typical Basidiomycete. In *Ascobolus,* failure of the progression due to incompatibility occurs at two distinct stages: (1) at the time of normal initiation of the sexual process; and (2) following plasmogamy—possibly a failure of dikaryosis (G. N. Bistis, personal communication). Under certain experimental conditions, plasmogamy between elements of the same incompatibility class occurs as in the normal cross. In *Podospora,* by contrast, sexual development is initiated without external stimulus and proceeds to the stage of plasmogamy, at which point development ceases until a fertilizing element of the opposite class is available (Esser, 1959a). There is a distinction, however, in the incompatibility systems involved in the two cases (see Section VI, A). In the Basidiomycetes, hyphal fusion between vegetative mycelia occurs in total disregard of mating type, and the discrimination due to incompatibility is exclusively intracellular and applies only after fusion to determine the product of interaction: a dikaryon, if compatible; heterokaryons of other types, if an incompatibility factor is common to the two mates. The above is the extent of the meager information available regarding the restrictions due to incompatibility.

The situation is somewhat brighter in respect to the regulation and coordination of stages in the sexual sequence, i.e., at the secondary level of control. Relevant information here is available from two very different lines of investigation: (1) the role of sexual hormones in the initiation and coordination of sexual activities in heterothallic species and (2) the effects of point mutations in the disruption of the normal sequence in homothallic species.

Sexual hormones in fungi were first demonstrated in *Mucor mucedo* by Burgeff in 1924 and since that time have been found in numerous forms scattered among the Phycomycetes and Ascomycetes. Since available reviews (Raper, 1952, 1957) give a comprehensive account of the early work in the field, we will confine our remarks here to recent developments and to the significance of hormonal control of sexual activities.

Only three cases have been investigated in sufficient detail to demonstrate the critical relationship between hormones and the several successive stages of the sexual process: *Achlya ambisexualis* and *A. bisexualis* of the biflagellate Phycomycetes (Raper, 1951 *et prior;* Barksdale, 1960), *Mucor mucedo* of the Mucorales (Plempel, 1957, 1960a,b; Plempel and Braunitzer, 1958; Plempel and David, 1960), and *Ascobolus stercorarius* of the discomycetous Ascomycetes (Bistis, 1956b, 1957; Bistis and Raper, 1963; Raper, 1957). The hormonal coordinating systems of these three plants are quite different in their operational details—quite

as diverse as are the morphological features of the three groups to which they belong. The three systems, however, have a number of features in common which indicate a basic pattern of control. In each of the three cases, hormones (*a*) initiate sexual activity, (*b*) induce sexual organ-formation, (*c*) control the differentiation of sexual organs, (*d*) determine the sequence of sexual organ-formation and activity, (*e*) quantitatively regulate the number of sexual organs formed, (*f*) serve as the agents of spatial orientation of sexual organs to permit plasmogamy, and (*g*) qualitatively restrict mating to closely related individuals. Furthermore, in each of the three cases, the hormones are secreted alternately by the two mates in a rigid sequence, each being liberated only after the achievement of the last preceding stage. The initiating and coordinating hormonal mechanism in each of these cases has evolved as a most efficient system of control to allow sexual activity only under circumstances that provide every possible guarantee for its consummation. Information about the chemical nature of these sexual hormones is fragmentary, and about the biochemical details of their activities, nonexistent. Current work seeks the identities of the initiating hormones in *Achlya* (A. W. Barksdale, personal communication) and in *Mucor* (Plempel, 1960b *et prior*). In the latter case, the zygophore-inducing hormone of *Mucor* has been isolated in chemically pure form, as the benzoic acid ester (Plempel, 1960b), and its identification appears imminent (Plempel, personal communication).

Demonstrations of sexual hormones in isolated stages of the sexual process have been reported in other forms including *Allomyces* of the aquatic Phycomycetes (Machlis, 1958a,b), the yeast *Saccharomyces* (Levi, 1956), and in the filamentous Ascomycetes *Neurospora* (Backus, 1939) and *Bombardia* (Zickler, 1934, 1952). Among these, the chemical study of the hormone sirenin, the chemotactic lure of male gametes in *Allomyces*, is well advanced toward identification (Machlis, 1958e, and personal communication).

That rigid control of the differentiative and sequential aspects of sexual reproduction, achieved via sexual hormones in the heterothallic forms described above, also operates in homothallic species is clearly shown by several studies relating genic mutations to the sexual process. Comparable results have been obtained in three homothallic species of the pyrenomycetous Ascomycetes: *Glomerella cingulata* (Wheeler, 1950, 1954, 1956b; Wheeler and McGahen, 1952), *Sordaria fimicola* (Olive, 1956, 1958, 1962; Carr and Olive, 1959; El-Ani and Olive, 1962; El-Ani *et al.*, 1961), and *Sordaria macrospora* (Esser and Straub, 1956, 1958; Heslot, 1958). In these three species, numerous genic mutations, either

spontaneous or induced by mutagenic agents, each renders the mutant strain self-sterile by the disruption of the normal developmental sequence at a characteristic stage. Such self-sterile, mutant strains, however, are cross-fertile via complementation of corresponding wild alleles in combinations that oppose nonallelic mutations. The developmental sequence is essentially the same in these species, and the results relevant to our present consideration can most easily be exemplified by the results reported for *S. macrospora* by Esser and Straub. Twenty-one (X-ray induced) mutant strains, 18 of which were self-sterile in isolation, were characterized as follows: 3 produced only vegetative mycelia; 3, ascogonia but no protoperithecia; 6, few to many protoperithecia but no perithecia; 6, sterile perithecia; and 3, fertile perithecia with nondehiscing asci. In all possible crosses between these mutant strains, 14 different genie mutations were identified. Not only were these 14 classes cross-fertile in all 91 possible crosses, but in many of the crosses were recovered normally developed selfed perithecia of one parental strain or of both parents. It is thus apparent that the developmental sequence is controlled by numerous genes, each of which is operative only in its proper turn.

Wheeler (1954) and Olive (1958, 1962) interpreted the results of studies such as those above as indicating a feasible origin of heterothallism of the incompatibility type by complementary mutations that either occurred in close proximity on the chromosome or were brought together into a compound locus via selection. El-Ani and Olive (1962) have recently reported two very closely linked, sterility mutations characterized by abortive asci and abortive ascospores, respectively. Crossing of these mutants results in normal development, but no recombination occurred among 504 asci tested. Such "balanced heterothallism" between defects at earlier stages in the developmental sequence would very closely mimic the native heterothallism of related forms, e.g., *Neurospora*. To postulate the origins of incompatibility in this way further suggests that incompatibility has independently originated many times in the fungi. Other students of sexuality in the fungi, the present authors included, feel that the very few variant types of incompatibility are evolutionarily related (as indicated by the detailed distribution of the different types) and that it is very unlikely that incompatibility originated in quite so casual a manner (Esser, 1963; Raper, 1959). Olive's (1958) "balanced heterothallism," however, is a positive step and could represent the first stage in a long evolutionary history. A more thorough understanding of the genetics and particularly of the biochemical mode of action of the incompatibility factors will be needed to determine

whether incompatibility is, in fact, basically a mutative disruption of normal sexual functions.

IV. Physiology

The fungi exist as a unique heterotrophic group, as they are incapable of photosynthesis. Their energy requirements are taken care of as saprophytes or parasites on dead or living plant or animal material. As foodstuffs, the fungi generally use very simple substances of low molecular weight, which they obtain through special physiological mechanisms by the decomposition of polysaccharides, proteins, nucleic acids, and other naturally occurring substances of high molecular weight into their component "building blocks." From these degradative products, which are also available to autotrophic plants for re-use, the fungi can then synthesize the necessary materials for growth and multiplication, as well as for the elaboration of characteristically fungal products.

Because of the physiological specialities, the fungi achieve biological as well as economic importance, and, because of the efficacy of their degradative processes, the fungi, together with the bacteria, serve as the scavengers of nature. Through their efficient synthetic processes, they serve industry (e.g., fermentation, cheese production) and medicine (e.g., antibiotics). The understanding of both of these physiological functions of the fungi is based, however, on a knowledge of nutritional physiology. Because of the restricted space at our disposal, we can present in this article only the most general outline of the physiology of the fungi. For full information, we refer the reader to the text books of Lilly and Barnett (1951), Hawker (1950), and Cochrane (1958).

A. General Nutrition

The study of nutritional physiology of the fungi is based on two premises: (1) on the use of synthetic nutrient media of which the components can be defined in qualitative and quantitative terms; and (2) on the use of pure cultures free of contamination from other organisms (bacteria, other fungi, etc.). During the past century, the nutritional requirements of a number of fungi have been investigated with the full observance of both of these criteria. It has been found, thereby, that the fungi require for their growth a carbon source, a nitrogen source, a number of minerals, and in some cases, vitamins.

Natural nutrient media, prepared from extracts of plant or animal materials, e.g., horse manure, malt extract, yeast extract, etc., are completely unsuited for nutritional-physiological studies since their contents cannot be defined. This does not lessen, however, the usefulness of these

natural media for routine investigations, e.g., crossing tests, maintenance of stocks, for which they still have far-reaching utility because of their low cost, availability, and ease of preparation. Also, the addition of agar-agar or gelatin to culture media should be avoided as much as possible, since the chemical compositions of these materials are not fully known.

1. Carbon Source

The carbon compound assumes a central place among the nutrients of fungi because of its utilization in two distinct ways: (1) it is important in the building of the structural and functional substances of living organisms—carbon represents about 50% of the dry weight of fungi—and (2) it is the unique, essential energy source of fungi. Carbohydrates and their derivatives are the common substrates; organic acids, fats, or acetate ions are but seldom utilized. Some fungi are also able to use carbon dioxide, but only in combination with organic carbon sources. It is assumed that the carbon dioxide is bound with pyruvic acid and other keto acids and that both play a role in the formation of amino acids (literature: Foster, 1949).

Since in metabolism of the fungi, the carbon material generally proceeds by way of hexose phosphate, it is understandable that D-glucose can be used by almost all fungi as carbon source. One exception is *Leptomitus lacteus*, which can satisfy its carbon requirement only by acetate ions or fatty acids (Schade, 1940). The use of other hexoses of mono-, oligo-, polysaccharides and their derivatives, therefore, depends on whether the fungus possesses the corresponding enzyme systems to transform these carbohydrates into glucose phosphate.

Cochrane (1958, pp. 68–73) has reviewed the carbon requirements of representative members of the three classes of fungi, Phycomycetes, Ascomycetes, and Basidiomycetes. From his tabulated summary, it follows that in certain fungi the utility of glucose is surpassed by that of other sugars. Since these cases, however, have not been physiologically analyzed in detail, they cannot override the general assumption that the assimilation of carbon proceeds by way of hexose phosphate. In all nutritional-physiological studies, it must further be borne in mind that many carbohydrates are hydrolyzed in the course of autoclaving of nutrient media. This is especially the case in media richly supplied with phosphate and having a pH of more than 6.

In general, the substrate-specificity of fungi for specific carbon sources is very marked, although it can vary between different geo-

graphical races of the same species. Many fungi are known, however, in which the formation of enzymes required for the degradation of specific carbon sources can be induced (Ammann, 1951; Christensen and Daly, 1951; Kylin, 1914; Young, 1918). In connection with inductive enzyme formation, the fungi in question are able to use, after an initial lag phase, nonspecific carbon sources in the normal manner. The substrate itself (induced enzyme formation), or substances that are either related or not related to the substrate, can function as inducing principles (Landman, 1954). By the same token, certain fungi can use, in a normal manner, "unusable" carbon sources, when to them the same small quantity of glucose is added (Lilly and Barnett, 1953). Glucose provides, in this case, the necessary energy for the initiation of growth and for the synthesis of induced enzymes. Substrate specificity in respect to the acceptability of carbon sources may be summarized as follows: cellulose and lignin are acceptable substrates for Ascomycetes and, especially, Basidiomycetes; the Phycomycetes, on the contrary, use mostly soluble, low-molecular-weight carbohydrates; the Chytridiales and certain biflagellate Phycomycetes constitute an exception, in that they are able to decompose cellulose.

2. Nitrogen Source

Nitrogenous compounds, like carbohydrates, are used in the formation of structural and functional substances, e.g., the structural unit of chitin, the wall substance of fungi (exceptions: yeasts and biflagellate Phycomycetes) consists of acetylchitosamine.

The fungi can be subdivided into four different classes on the basis of their nitrogen requirements (Robbins, 1937; Steinberg, 1939, 1950). This subdivision is based on the following sequence of nitrogen sources: atmospheric nitrogen, nitrate nitrogen, ammonia nitrogen, and organic (particularly amino) nitrogen. The representatives of class 1 are able to use all four components; those of class 2 cannot use atmospheric nitrogen, but they can use the nitrogen in the three other groups; those in class 3 can use only ammonium and organic nitrogen; whereas those in class 4 are limited to the utilization of organic nitrogen.

In contradiction to the findings of previous authors (reviews: Allison *et al.*, 1934; Duggar and Davis, 1916; Foster, 1949; Goddard, 1913; Senn, 1928), it is now doubtful that any true fungi can assimilate gaseous nitrogen under any circumstances. An attempt to confirm with isotopes the competence of nitrogen fixation ascribed to the mycorrhizal fungus *Phoma* has led to contradictory results (Tove *et al.*, 1949; Wilson, 1951).

Lilly and Barnett (1951) give a compilation of fungi belonging in

the individual classes. In the application of Robbins' classification, it is often overlooked that an entire series of "fungi"—the free-living nitrogen-fixing bacteria—can utilize N_2-nitrogen as a sole nitrogen source (Yoshii, 1935; Fries, 1955; Talley and Blank, 1942; Morton and MacMillan, 1954; Quantz, 1943).

The overlapping of nitrogen requirements of individual classes is accounted for by the fact that the nitrate- and, especially, nitrite-nitrogen first enters into metabolism after reduction to ammonium-nitrogen, which then serves as a point of departure for the synthesis of organic nitrogen.

Proteins and their degradation products serve, in the main, as organic nitrogen sources. Most of the fungi have an ample supply of enzymes for the hydrolysis of proteins and especially for the decomposition of peptides. Protein-nitrogen is taken up by fungi only in the form of the constituent amino acids or the corresponding amides.

Insofar as the amino acids are not immediately broken down in metabolism, they are deaminated and further used as ammonium (Barton-Wright, 1952; Thorne, 1950). Certain fungi exhibit marked specificities in respect to amino acids (Leonian and Lilly, 1938), comparable to their specificities for carbon sources. Nucleic acids and their degradation products (Wolf, 1953, 1955) and urea may be used as additional organic nitrogen sources.

3. *Minerals*

The fungi require relatively large quantities of phosphorus, calcium, sulfur, and magnesium (literature: Foster, 1949; Cochrane, 1958). These substances are administered in a concentration of the order of 10^{-3} *M* as mono- or dibasic phosphates or in a concentration of 10^{-4} *M* as sulfates. As trace elements, iron, zinc, copper, manganese, and molybdenum are added in most cases to the nutrient medium in ionic form in a concentration of the order of 0.1 part per million, with the exception of molybdenum, of which only ca. 0.0001 p.p.m. is required. The mycelial growth rate, in a series of species of all classes of fungi, is positively influenced by Ca^{++} (5–20 p.p.m.).

A requirement for cobalt has only been proved in those fungi that produce vitamin B_{12}.

The utilization of minerals is both constitutive, e.g., phosphorous for nucleic acids and sulfur for proteins, as well as functional, e.g., metal ions as activators or active centers for enzymes. In certain cases, it is possible for a mineral to be substituted for a closely related one, e.g., potassium for sodium (Boysen-Jensen, 1932; Hirata *et al.*, 1954).

B. Vitamins

The fungi require, as do all other organisms, small quantities of vitamins for their growth, reproduction, and other vital processes. Only a very few fungi (list: Lilly and Barnett, 1951) are prototrophic for all vitamins; most fungi are partially or fully auxotrophic for one or several vitamins.

Since, in general, only small quantities of the appropriate vitamins are necessary for the vitamin-auxotrophic fungi, the determination of prototrophy is very difficult, because, in the nonsynthetic components of nutrient media, and especially in the sugars, traces of vitamins are regularly present.

The most commonly encountered vitamin-deficiency among the fungi is that for thiamine (vitamin B_1) and especially for the pyrimidine moiety of this growth substance. Auxotrophy for the thiazole moiety is far more rarely encountered (Robbins and Kavanagh, 1942). Auxotrophy for biotin (vitamin H, coenzyme R) is also widespread and is generally coupled with thiamine auxotrophy. Far more seldom found are auxotrophies for pyridoxine (vitamin B_6, adermin), riboflavine (vitamin B_2, lactoflavine), nicotinic acid, inositol, *p*-aminobenzoic acid, pantothenic acid, pteroylglutamic acid (vitamin B_c), and vitamin B_{12}. In addition to the vitamin deficiencies that have been reported as occurring in isolates from nature, many have been experimentally induced as genic mutations.

Since vitamin autotrophy varies considerably within the individual taxa of the fungi, no strict correlation can be found between vitamin needs and systematic position of individual species. Also the ecological groups, such as soil fungi, water fungi, saprophytes of plant, and, especially, animal parasites, reveal no common pattern of vitamin deficiencies, if one disregards the almost universal thiamine auxotrophy of the Agaricaceae, the members of which can be assigned to the same ecotype as wood-destroying and humus-forming fungi.

V. Basic Genetics

In general, it is of little use to consider the genetics of a group of organisms in detachment from the basic science of genetics. Our treatment of fungal genetics will accordingly concentrate upon the contributions of fungal studies to the science of genetics and the role of genetics in fungal studies.

A few remarks relating to the early history of the study of fungal genetics seem fitting at this point. Although Blakeslee (1904, 1906) first

carried out crosses in the Mucorales and also made preliminary analyses of the progeny of crosses, it was Burgeff (1912, 1914, 1915) who first made a systematic analysis of a fungus and demonstrated that a pair of alleles is responsible for the occurrence of two mating types in heterothallic species of the Mucorales. The weight of fungal genetics, however, was transferred soon thereafter to the Basidiomycetes. This was primarily determined by the personality of Hans Kniep who, from 1920 until his untimely death in the early 1930's, concerned himself with the genetics of the sexuality of this fungal group. His comprehensive review, *Vererbungserscheinungen bei Pilzen* (1929) and his *Sexualität der niederen Pflanzen* (1928) remain the classics of fungal genetics. The flourishing of fungal genetics, which has brought it to its present-day significance, began with the work of Dodge (1927, 1928a, 1929) on the Ascomycetes. The description of the life history of and the cultural conditions for the now well-known species *Neurospora crassa* by Dodge, and the original examination of the genetics of this fungus by Lindegren (1932, 1933), created the basis for the study of formal genetics as well as for the investigation of gene function in the fungi. In later years, numerous comprehensive reviews concerning limited aspects of fungal genetics have appeared, and these reviews will be cited in the appropriate places. Consideration of fungal genetics is generally minimized in genetics textbooks; textbooks with a microbiological bias, however, provide an important exception: Wagner and Mitchell (1955), McElroy and Glass (1957), Kaudewitz (1957), Pontecorvo (1958), Anfinsen (1959), Hayes and Clowes (1960), and the reviews of Lindegren (1948) and Wheeler (1958). Any deficiency in this special field has largely been dispelled by the recent publication of an excellent, comprehensive book "Fungal Genetics" by J. R. S. Fincham and P. R. Day (1963).

Genetic considerations here will emphasize two major aspects of fungal genetics as it relates to the whole of the science of genetics: (1) fungal contributions to basic genetics, and (2) the basic genetics of features peculiar to or especially prominent in the fungi. In the former case, both the general characteristics of fungi, e.g., short life history, easy and rapid culture, haploid vegetative phase, as well as features peculiar to fungi, e.g., ordered tetrads and heterokaryosis including dikaryosis, have often made a variety of fungi nearly ideal, or even occasionally indispensable, materials for the study of many basic genetic phenomena. In the latter case, the basic nature of many fungal features, e.g., incompatibility, heterokaryosis, parasexuality and somatic recombination, and pathogenicity, have been tremendously illuminated through the careful study of their genetic characteristics.

The fungi have shared with bacteria and viruses a commanding role as suitable materials for the genetic research which, during the past few years, has brought such penetrating insight into the physical and chemical nature of genetic materials and genetic phenomena. In this period of intensive and rewarding investigation, studies with fungi have made very significant contributions in each of the four major problem areas of basic genetics: factor analysis, biochemical pathways, the organization of genetic material, and extrachromosomal inheritance.

A. Factor Analysis

Factor analysis in the fungi provides, in comparison to the classic materials of genetics, i.e., *Drosophila*, maize, mouse, etc., two advantages: (1) the vegetative stage of most fungi is haploid and (2) tetrad analysis is possible in many cases, i.e., the four products of a meiotic division can be separately grown and genetically analyzed.

1. Tetrad Analysis

The analysis of tetrads, which is possible in many Eumycetes, permits conclusions to be drawn regarding the successive division of the chromosome and especially of the segregation of genes in the daughter nuclei during the course of meiosis. In the eight-spored Ascomycetes, spores originate in pairs from each of the four products of meiosis. The spores of the four-spored yeasts and of the Basidiomycetes each contains only one of the four nuclei originating in meiosis.

A primary requisite for tetrad analysis is a thorough understanding of the cytological course of meiosis. It is generally acknowledged today that crossing over occurs in the four-strand stage, in the prophase of meiosis I, and it is further known that the centromeres of homologous chromosomes are regularly prereduced, i.e., they separate in meiosis I (Ludwig, 1937, 1938; Knapp, 1937, Ryan, 1943; Tatum and Perkins, 1950). Among animals and higher plants, certain exceptional cases are still cytologically unclear (Rieger and Michaelis, 1958). Segments of chromosomes that lie distal to a point of chromatid exchange with reference to the centromere, however, are postreduced, i.e., they divide at meiosis II. A gene is accordingly postreduced when a crossover occurs between its locus and the centromere and is prereduced when between its locus and the centromere either no crossing over occurs or crossing over occurs at one or several additional points to cancel the effects of recombination.

Especially advantageous for tetrad analysis are certain Ascomycetes, in which the spores are linearly ordered in the ascus as a result of their

mode of origin during meiosis. Here belong the well-known genetic materials *Neurospora crassa*, *N. sitophila*, *Bombardia lunata*, *Sordaria macrospora*, and *S. fimicola*.

Suitable mutations, in which the mutant characteristics are evident in the ascospores, e.g., spore color, permit very rapid tetrad analysis in certain cases: *Sordaria fimicola* (Bistis and Olive, 1954; Olive, 1956), *S. macrospora* (Heslot, 1958; Esser and Straub, 1958), *Ascobolus immersus* (Rizet, 1958).

The outstanding reviews of tetrad analysis in fungi are those of Whitehouse (1942), Papazian (1952a), Perkins (1953, 1955), Bennett (1956), Shult and Lindegren (1956).

a. Ordered tetrads. In ordered tetrads, the centromere can be used as a marker because of its constant prereduction. This makes possible the determination of the relative position of a given locus to its centromere in a single-factor cross: one-half the frequency of its postreduction corresponds to its distance from the centromere as expressed in conventional crossover, or Morgan, units, since a crossover in the four-strand stage between the gene and its centromere yields two parental products and two recombinants between gene and centromere.

The localization for all genes not closely linked to their centromeres requires, however, a correction for multiple crossovers and interference (see page 185) by so-called linkage functions (see page 188).

Of even greater utility in tetrad analysis than single factor crosses, however, are two-factor crosses, which here deserve consideration in some detail. Such crosses permit the genetic estimation of meiotic events of two chromosomal regions that are bounded by both loci and their centromeres.

Among the asci from a two-factor cross (e.g., $a^+b^+ \times ab$), seven different ascus types are possible as a consequence of the pattern of reduction of both gene pairs and of the combination of four nuclear types (Fig. 4). These seven types can be catagorized into classes in two different ways: (1) according to the pattern of reduction of both gene pairs into groups A (both gene pairs prereduced), B (both gene pairs postreduced), and C and D (either gene pair prereduced, the other gene pair postreduced), and (2) according to the genotypes of the tetrad nuclei as groups I (parental types), II (recombinant types), and III (parental and recombinant types). Groups I and II are termed parental and nonparental ditypes, respectively, and group III is termed tetratype.

By the determination of the frequency of postreduction, tetrad analysis of a two-factor cross makes possible, by the scheme of Fig. 4,

the location of both genes in respect to their centromere or centromeres and, in certain cases, permits an examination of the phenomenon of interference.

i. Frequency of postreduction. The frequency of postreduction for the gene pair a^+/a corresponds to the sum of the relative frequency of types 3, 4, 5, and 6 in Fig. 4. The corresponding value for the gene pair b^+/b results from the sum of the relative frequency of groups 3, 4, 5, and 7.

ii. Linkage. When the two gene pairs are not linked and are located in different chromosomes, there occur the seven tetrad types of Fig. 4

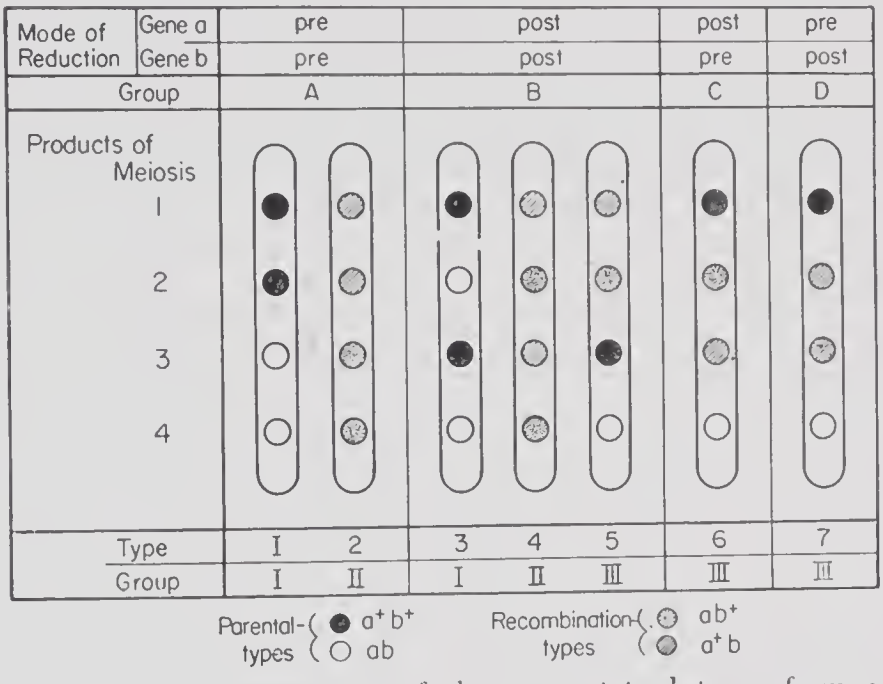

FIG. 4. Schematic representation of the seven tetrad types from a two-factor cross, $a^+b^+ \times ab$. Instead of eight spores, only the four meiotic products are shown. For details, see text.

by the chance combination of pre- and postreduction of the gene pairs. For linked genes, this randomness does not obtain, since postreduction of the two gene pairs is no longer independent.

The following criteria for linkage can be used. (1) Deviation of observed tetrad values from those expected from independent postreduction of the two genes. In the absence of linkage, the relative frequencies of tetrads of groups A–D correspond to the products of the frequencies of prereduction of both genes (group A), of postreduction of both genes (group B), and of prereduction of either gene and postreduction of the other (groups C and D). (2) Deviation from a 1:1 ratio for parental and recombinant types in group A. (3) Deviation from a 1:1 ratio for parental and recombinant types in group B occurs only

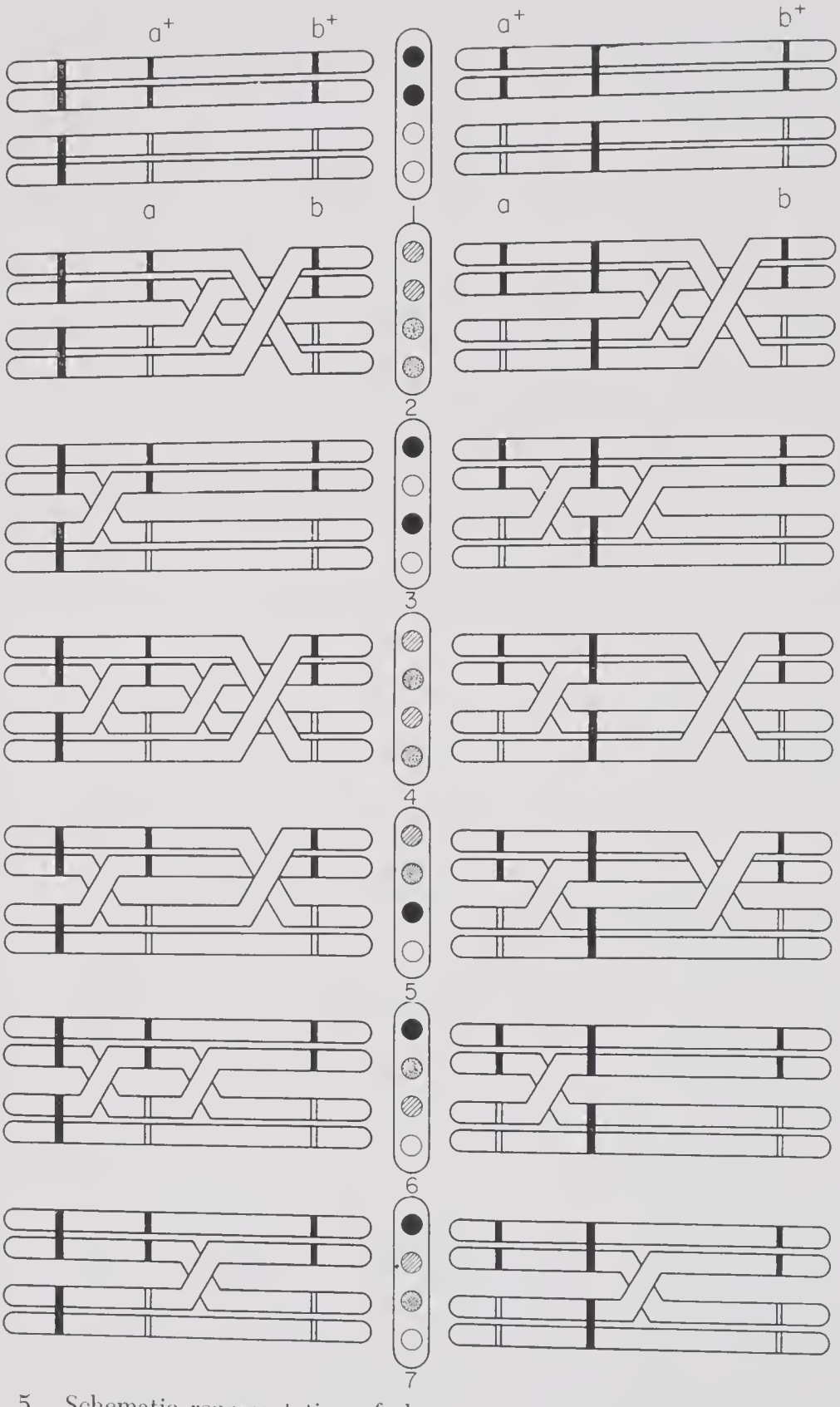

FIG. 5. Schematic representation of the minimal number of crossovers between two linked genes that lead to the formation of the seven different ascus types of a two-factor cross (see Fig. 4). On the left, both genes are on the same chromosome arm; on the right, the two genes are on different sides of the centromere. Genotypes are identified as in Fig. 4.

if the two genes are located in the same chromosome (Fig. 5); this criterion thus provides the means of determining whether the two genes are located on the same arm or on different arms of the same chromosome. (4) Similarly, in the relative frequency of types 3, 4, and 5 of group B, a variation from 1:1:2 ratio indicates linkage on the same chromosome arm. If the two genes lie on different chromosome arms, the following types originate in double crossovers (Fig. 5): type 3, in two-strand double crossovers, type 4, in four-strand double crossovers, and type 5, in three-strand double crossovers. Neglecting chromatid interference, the proportion of the three types of double crossovers should occur in the ratio 1:1:2. Conversely, deviation from this ratio indicates chromatid interference, once it is established, through other mapping investigations, that the two genes lie on different sides of the centromere. Finally, (5) the value of recombination can still be established for linkage of less than 0.5 units. Variations between the recombining values and the locations of the genes, as ascertained by their frequencies of postreduction, indicates chromosome interference.

In multiple-factor crosses, all relevant genes taken two at a time can also be analyzed by the scheme of Fig. 4.

As with ordered tetrads, asci which form only four linearly ordered binucleate spores can also be analyzed if the cytologieal events that lead to spore formation are known. In this group belong *Neurospora tetrasperma* (Dodge, 1928b), *Gelasinospora tetrasperma* (Dowding, 1933; Dowding and Bakerspigel, 1956), *Podospora anserina* (Rizet and Engelmann, 1949; Esser, 1956). Especially in *Podospora anserina*, the genetic investigations of Rizet and Engelmann and the cytological work of Franke (1962) have shown that the two nuclei of each spore are non-sister nuclei of postmeiotic mitosis: each spore contains one daughter nucleus of each of two of the four products of meiosis (Fig. 6a). Since in these four-spored Ascomycetes, a pair of uninucleate spores occurs instead of a single binucleate in about 1–2% of the asci, half-tetrad analysis is feasible if a pair of uninucleate spores occur in the same half of the ascus (Fig. 6b). Tetrad analysis is possible if each half of the ascus contains a pair of uninucleate spores (Fig. 6c).

b. Unordered tetrads. In the asci of yeasts (exception: Hawthorne, 1955), many Euascomycetes (*Glomerella*, *Aspergillus*, *Ascobolus*), and in the basidia of the basidiomycetes, the spores of the tetrad are not linearly ordered. Since in unordered tetrads the centromere fails as a marker, one gene more than in ordered tetrads is required to provide the same information. The frequency of postreduction cannot be directly determined, but it has been determined from multiple-factor crosses by

the use of appropriate methods formulated by Whitehouse (1949b, 1950, 1954, 1957), Perkins (1949), Papazian (1952b), and Bistis (1956a). The classification of ditype and tetratype tetrads is possible in two-factor crosses (groups I, II, III, cf. page 181). The criteria of linkage are the ratios of parental and nonparental ditypes and the frequency of recombination.

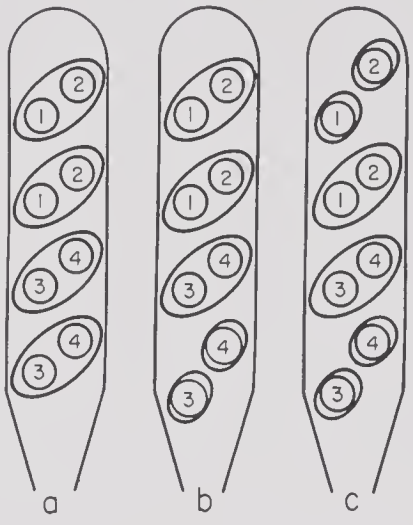

FIG. 6. Schematic representation of the distribution of the four products of meiosis in the asci of *Podospora anserina*. The meiotic products are designated by figures drawn in the nuclei of the spores. For details, see text. From Esser (1959a).

2. *Random Analysis*

The analysis of single meiotic products provides only the determination of recombination. Random analysis is used either to avoid the laborious isolation of tetrads when large samples of progeny are needed, e.g., for the analysis of fine structure of a genetic region, or in those fungi (most of the Phycomycetes) in which the spores, when ripe, no longer remain together in tetrads.

3. *Interference*

The outstanding utility of tetrad analysis for the study of interference has long been recognized (Muller, 1916). Interference is defined as the mutual influence of adjacent exchanges during crossing over, and this influence is expressed as a failure of the random occurrence of subsequent crossovers. In chromatid interference, one crossover effects a nonrandom involvement of chromatids in the further course of exchange. In chromosome interference, this influence is independent of the chromatids and affects only the probability of additional exchanges. Positive interference results in a reduction of total frequency of crossing over, whereas negative interference results in an increase.

a. Chromatid interference. Chromatid interference results in a deviation in the frequencies of 2-strand, 3-strand, and 4-strand double crossovers (cf. also Fig. 5) from a 1:2:1 ratio. Positive interference thus increases 4-strand double crossovers, and negative interference increases 2-strand double crossovers. Prerequisite for the determination of chromatid interference, according to this criterion, is the validity of "sister-strand-verbot" (Morgan, 1933; Weinstein, 1936), according to which exchanges between sister chromatids do not occur. Such exchanges, however, have been detected by Schwartz (1953) in ring chromosomes of maize.

In *Neurospora crassa*, negative interference was found which, at least in linkage-group I (mating type locus), encompassed the centromere (Lindegren and Lindegren, 1937, 1939; Whitehouse, 1942). Other regions of the same chromosome, however, seem to show essentially no negative or positive interference (Lindegren and Lindegren, 1942). Howe (1956) and Stadler (1956a) found no chromatid interference in the same material, and Perkins (1955) found no significant deviations from 1:2:1 ratio of double crossovers in intervals of 10–23 crossover units. These findings have cast doubt on the existence of chromatid interference in *Neurospora*. Through the extensive investigations of Strickland (1961) and Pateman (1960), however, chromatid interference in linkage group V was demonstrated in this fungus. In order to explain the contradictory results concerning the existence of chromatid interference in *Neurospora*, Strickland has proposed the adoption of the "two-phase model" of Schwartz (1953, 1954), which envisages a variability in sister-strand exchange.

Negative chromatid interference has been demonstrated in *Neurospora sitophila* (Whitehouse, 1956), *Saccharomyces cerevisiae* (Shult, 1959; Hawthorne and Mortimer, 1960), *Aspergillus nidulans* (Pritchard, 1955, 1959), *Ascobolus immersus* (Rizet *et al.*, 1960a). According to the data of Pritchard on *Aspergillus*, localized chromatid interference occurs in this material: exchanges within one cistron causes an increase of exchange in adjacent regions.

Positive chromatid interference has not yet been described.

b. Chromosome interference. The phenomenon of chromosome interference has been known for a long time and has been demonstrated in many materials (Reck, 1948; Rizet and Engelmann, 1949; Kuenen, 1962b; Ludwig, 1938; Papazian, 1952a; Barratt *et al.*, 1954; Desborough and Lindegren, 1959; Shult, 1959). In the absence of chromosome interference, there exists a random, Poisson, distribution of two-strand and multiple-strand crossovers. Significant deviations from this distribution

reveals chromosome interference and quantifies its expression in the "coefficient of coincidence." The coefficient of coincidence is equal to the quotient of the number of demonstrated double crossovers and the number expected from the Poisson distribution.

Negative chromosome interference across the centromere has been demonstrated in *Neurospora crassa* (Lindegren and Lindegren, 1937, 1942; Whitehouse, 1942; Papazian, 1952a). Positive chromosome interference can be found only in long chromosomal segments, the size of which allows the random appearance of several crossovers. The advantage of tetrad analysis for the determination of interference lies in the fact that it is possible, by the use of a single marker gene, to get an insight into the process of recombination. Since, in the absence of interference and because of the random distribution of crossovers, the frequency of postreduction of single genes does not exceed the value of 0.667 (Mather, 1935, 1938; Rizet and Engelmann, 1949; Papazian, 1951), the frequency of postreduction in single-factor crosses can reveal, under certain circumstances, the occurrence of chromosome interference. Certainly, the existence of chromatid interference is not completely excluded with this gross method.

4. Gene Mapping

Since the investigations of Morgan on *Drosophila*, it has been known that the genes are linearly ordered on the chromosome. The sequence of genes can, by determination of recombination, be shown in ordered tetrads by the frequency of postreduction and in unordered tetrads by the frequency of the different types of tetrads.

It is clear from the diagram of crossover sequence in Fig. 5 that tetratype frequency in unordered tetrads corresponds to the frequency of postreduction, if one of the two genes is considered as a marker for its respective centromere. Linkage values, expressed in crossover units, are equal to one-half the frequencies of postreduction or recombinant tetrads.

These values are only additive, however, if one considers regions of a few crossover units, in which the possibility of multiple crossovers and total interference can be excluded. Linkage maps, which are constructed without attention to these criteria, permit only an estimate concerning the sequence of genetic factors. For the determination of map distance between genes, which is defined as the percentage recombination by crossing over between them, the formulation of a mapping function is required. With the help of this function, a correction is established for the values of recombinations, postreduction, and tetrad

types relative to multiple crossovers and interference. Single-strand mapping functions permit the conversion of recombining values to map distances. Tetrad mapping functions are used for the correction of data relating to postreduction or tetratype tetrads.

Barratt *et al.* (1954) formulated such mapping functions for *Neurospora crassa*. They considered theoretically, first of all, a series of single-strand and tetrad mapping functions for ten different intensities of crossover interference, with values of coincidence ranging between 0 and 1. The following assumptions were made for the formulation of these

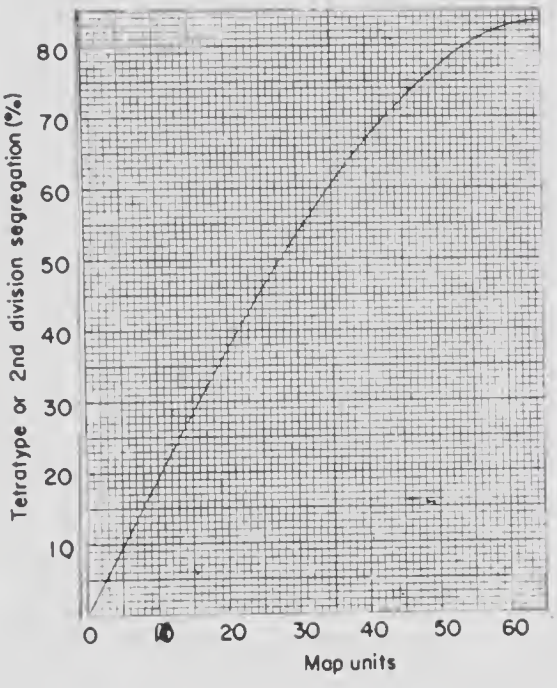

FIG. 7. Tetrad mapping function curve with interference corresponding to $K = 0.2$. Based on a model of Barrat *et al.* (1954).

functions: (1) chromatid interference does not occur within a chromosome arm; (2) the probability with which no crossover occurs is not influenced by interference; and (3) positive interference changes the frequency of one, two, and multiple crossovers in a regular manner. By combining the single-strand and tetrad mapping functions, for each coincidence value there was revealed a third function, which expressed the dependence of values of recombination upon the frequencies of postreduction and, especially, tetratype tetrads. In this coordinate system, Barratt *et al.* applied the relevant data on postreduction and tetratype frequencies and values of recombination from the literature. The points plotted from experimental data gave good agreement with the theoretical function for coincidence values of $K = 0.2$ to 0.3. The method is considered definitive for $K = 0.2$, since this degree of inter-

ference appears to occur also for *Drosophila*, which has been more adequately investigated. With the help of single-strand and tetrad mapping functions for the coincidence value of 0.2 (Fig. 7), they carried out the extensive mapping of *Neurospora crassa*. The data were later essentially confirmed (Stadler, 1956b; Perkins, 1959; Perkins and Ishitani, 1959; Maling, 1959; Strickland *et al.*, 1959). More than 130 loci have now been localized. Rifaat (1958) also gave a method for the mapping of *Neurospora* data.

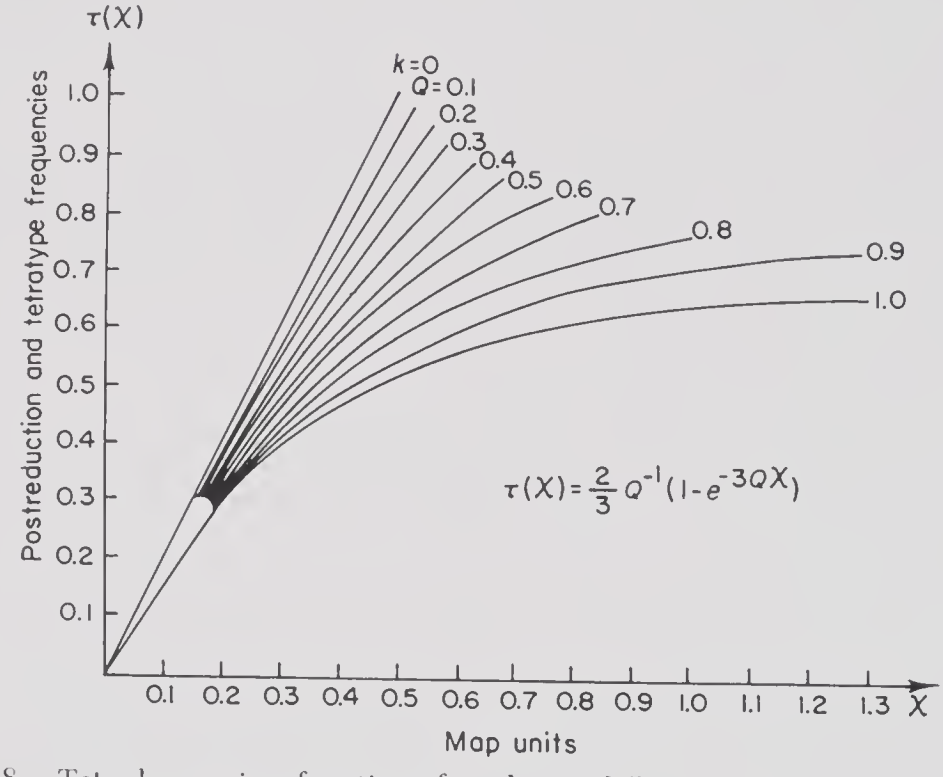

FIG. 8. Tetrad mapping functions for eleven different degrees of interference: $K = 0$, total interference; $Q = 0.1$ to 0.9, positive interference; $Q = 1$, no interference. From Kuenen (1962b).

An additional mapping model for tetrads was formulated by Kuenen (1962a). The assumptions of this model correspond essentially with those of Barratt *et al.* (1954). These assumptions are related by Kuenen, however, not only to the region which, by chance, happened to be in a linkage group between two markers, but also in the total region of the genetic structure in which interference regularly occurs (the interference region). In this way were formulated the mapping functions diagrammed in Fig. 8.

Mapping of *Saccharomyces cerevisiae* has been done by Lindegren and his associates (Shult and Lindegren, 1955, 1956 Lindegren *et al.*, 1959; Desborough and Lindegren, 1959). Extensive mapping in the yeasts was also undertaken by Hawthorne and Mortimer (1960), who

were able to arrange more than 70 genes in ten linkage groups. For one linkage group, of which the genes lie in a single chromosome arm, a chromosome interference of $K = 0.34$ was calculated.

Mapping in other fungi has been carried out in numerous materials including *Sordaria fimicola* (El-Ani *et al.*, 1961), *Sordaria macrospora* (Heslot, 1958), *Glomerella cingulata* (Wheeler, 1956a), *Ascobolus immersus* (Rizet and Lissouba, 1960; Lissouba, 1960), *Podospora anserina* (Kuenen, 1962b), *Schizophyllum commune* (Raper and Miles, 1958), *Coprinus lagopus* (Lewis, 1961a; Day, 1960a), *Schizosaccharomyces pombe* (Leupold, 1958b), *Venturia inaequalis* (Boone and Keitt, 1956), and *Aspergillus nidulans* (Käfer, 1958).

5. *Factors Influencing Segregation Patterns in Tetrads*

The pattern of segregation in tetrads, which is determined by crossing over and finds its expression in the frequency of postreduction or the frequency of tetratype tetrads, can be influenced by genetic factors as well as by nongenetic factors.

Influence upon crossing over of the genotypes of the particular partners of a cross has been found by Stadler (1956a), Perkins (1959), and Frost (1961) in *Neurospora*, and by El-Ani *et al.* (1961) in *Sordaria fimicola*, and by Raper *et al.* (1958a, 1960) in the mating type factor of *Schizophyllum commune*.

Far more data are available, however, about the influence of external conditions, especially the influence of temperature: Hüttig (1931, 1933) in *Ustilago* spp.; Wülker (1935) in *Neurospora sitophila*; Zickler (1934, 1937) in *Bombardia lunata*; Rizet and Engelmann (1949) and Monnot (1953) in *Podospora anserina*; Stadler (1956a) and Rifaat (1956, 1959) in *Neurospora crassa*; Bistis (1956a) in *Ascobolus stercorarius*; Olive (1956) in *Sordaria fimicola*; Heslot (1958) in *Sordaria macrospora*; and Lissouba (1960) in *Ascobolus immersus*. In all these investigations, most of which are based on tetrad analysis, it has been shown that certain genes react to increased temperature with an increase in the frequency of postreduction whereas others react with a decrease. A satisfactory interpretation of these findings has not been proposed.

6. *Anomalous Segregations and the Analysis of the Fine Structure of Genes*

Polarized segregation, i.e., different frequencies between symmetric and asymmetric classes in postreduced asci, was found in *Bombardia lunata* (Zickler, 1934; Catcheside, 1944; Mathieson, 1956) and nonrandom distribution of asci of parental, nonparental, and tetra types

was reported by Mitchell (1957) in *Neurospora crassa*. Besides these isolated observations of segregational anomalies of individual ascus types, far more frequent segregational anomalies within tetrads have been demonstrated. This applies especially to the so-called 3:1 segregation, i.e., in one-factor crosses, three products of meiosis carry the genotype of one parent and only one product of meiosis carries the genotype of the other parent. In the eight-spored Ascomycetes, 3:1 segregation is manifested in the ascus naturally as a 6:2 segregation. It has come into general use to designate this anomaly as "conversion" without reference to the original introduction of the term by Winkler in 1930 (Bonner, 1956; St. Lawrence and Bonner, 1957).

The phenomenon of conversion was first observed in tetrads by Zickler in *Bombardia lunata* (1934). Unfortunately, these data have been wholly overlooked in the literature. The problem of conversion was again taken up by Lindegren and associates in *Saccharomyces cerevisiae* (Lindegren, 1953, 1955; Lindegren *et al.*, 1956; Ogur and St. John, 1956; Pittman and Pedigo, 1956). Lindegren's data concerning anomalous segregation of 3:1 and 4:0 in yeast tetrads were met from the first with skepticism and controversy. Thus Winge and Roberts (1954a, b, 1957) were able to show that abnormal segregations occurred in the asci of yeast as a consequence of postmeiotic mitoses which led to the formation of 5- to 8-nucleate asci with, however, only four spores, which were partially heterozygous. Another possible "explanation" of conversion in yeast is polyploidy, which also has been frqeuently demonstrated. To the present time, tetrad analysis carried out by Lindegren *et al.* (1956) and Roman (1958) on *S. cerevisiae* and by Leupold (1958b) on *Schizosaccharomyces pombe*, however, leave no doubt of the reality of conversion in yeast.

Investigations of Mitchell (1955, 1956) paralleling in *Neurospora crassa* the studies in yeast on conversion demonstrated the phenomenon in this material. Mitchell constantly found a few prototrophs in crosses of allelic auxotrophic mutations (e.g., 103 prototrophs among 66,959 isolates for the pyridoxineless locus). Since it could be shown through tetrad analysis that, in the asci with conversion, the double mutations expected according to the Mendelian scheme were absent, crossing over, in the classic sense, cannot be responsible for the formation of prototrophs.

Stimulated by the experiments of Mitchell, a series of other authors have since succeeded in demonstrating gene conversion in *Neurospora crassa* as well as in other fungi, both through random analysis as well as through tetrad analysis and through judicious selection of marker genes, which were closely linked with the investigated loci: de Serres

(1958), Giles *et al.* (1957a), St. Lawrence (1956), Case and Giles (1958a), Freese (1957a, b), Pateman (1958), Suyama *et al.* (1959) Weijer (1959), and Stadler (1959) in *Neurospora crassa;* Rizet *et al.* (1960a) in *Ascobolus immersus;* Olive (1959) in *Sordaria fimicola;* and Forbes (1956) and Pritchard (1955) in *Aspergillus nidulans.* For the clarification of the phenomenon of conversion, Lindegren invoked the theory first set forth by Winkler, according to which interaction between allelic genes in a heterozygote, *A/a*, can bring about the conversion of a dominant allele to recessive or the converse. Mitchell proposed a copy-choice model, in which a chromatid could be reduplicated twice, or not at all. St. Lawrence assumed controlling elements, such as are known in maize, as the basis for the occurrence of conversion. Additional models for the basis of conversion have been proposed by Roman (1956), H. K. Mitchell (1957), Freese (1957b), St. Lawrence and Bonner (1957), and Rizet and Lissouba (1960). A decision for one or another of the models, which mostly depend upon copy choice, is, unfortunately, as yet impossible. It is equally clear that gene conversion within single loci does not occur through classic crossing over. These investigations show astonishing parallelism to the observations of Benzer (1955) on bacteriophage concerning the fine structure of a gene locus, since factor analysis of crosses between allelic mutants show that these can be linearly ordered within a locus by the frequency of prototrophs. In such a way, maps for fine structure of single genes have to date been constructed for different loci of the materials listed above.

The analysis of the phenomenon of conversion has lead to a revision of the concept of the gene, since the allelic concept, according to which the alleles should be located in the same position in homologous chromosomes, is no longer tenable. Consideration should be given to the substitution for the gene concept in organisms with true chromosomes of the terminology proposed by Benzer (1957) for phage genomes: *recon, muton,* and *cistron,* which are defined as the smallest units of recombination, mutation, and physiological function, respectively.

Rizet and co-workers (Rizet and Lissouba, 1960; Rizet *et al.,* 1960b; Lissouba, 1960) have made use, for fine structure analysis of *Ascobolus immersus,* of the possibility implicit in mutations for spore color, which permit in a short time the tetrad analysis of very large numbers of asci. Approximately 2000 mutations with white spore color can be classified into twenty different gene locations. Through tetrad analysis of fine structure of two of these loci, it was found that each of the two loci consisted of at least two genetic regions, which, through crossing over, are separable one from the other. Within each region, no crossing over,

only conversion, is observed. The individual mutant sites, which in each region are linearly ordered, each shows a polarity relative to its pattern of conversion. This is expressed in the following way: in conversion tetrads from crosses of two mutant alleles, the parental character lying to the left is represented by two products of meiosis and the other parental character only with one product of meiosis, while the fourth meiotic product is represented by a wild-type nucleus that originated through conversion. The polarization can also occur from the other side. The existence of this *polaron*, the linear structural unit, is in good agreement with the proposed linear-duplex model of chromosome structure of Freese (1958) and Taylor (1958).

A further example for the use of mutations of spore color in the analysis of abnormal segregation is given by the investigations of Olive (1959) on *Sordaria fimicola*. Olive found, in this 8-spored Ascomycetes, 5:3 and 7:1 segregations in addition to the 6:2 segregation already known. These types of conversions serve as confirmation for the cytological observations made by Taylor that each chromatid consists of two functional subunits. 5:3 segregation was also found in *Ascobolus* by Rizet *et al.*

Finally, one can say that factor analysis in fungi has confirmed previously obtained genetic information based on the study of *Drosophila* and other genetic materials, and much new information concerning the fine structure of the gene has been gained from tetrad analysis. The differences between nucleus-containing and non-nucleus-containing organisms have been decreased, and the basic principles of a unified genetics of both groups have been created. Further results of factor analysis form the basis for an examination of the physiology of the gene, to be considered in the following section.

B. Biochemical Pathways

Studies concerning the physiological activity of genes go back to the investigation of Garrod (1909, 1923) on the inheritance of human alcaptonuria and the research on the pigmentation of insects (Kühn, 1941, 1948; Ephrussi, 1942; Butenandt *et al.*, 1940, 1942; for literature, see Beadle, 1945a). These investigations have shown that genes bring about their effects in specific metabolic steps. Concepts relating to the mechanism of genic activity have, in large part, been brought about by the extensive studies on auxotrophic mutations in *Neurospora crassa* which were initiated by Beadle and Tatum. Since the results of these researches constitute at present a large portion of many textbooks and the basis for numerous reviews (Tatum and Beadle, 1942b; Beadle,

1945a, b, 1946, 1948, 1955, 1957, 1959; Tatum, 1944, 1959; Bonner, 1946a, 1951; Horowitz, 1950, 1951; Horowitz *et al.*, 1945), we will give only a summary of this work here. The Ascomycete *Neurospora crassa* has proved to be eminently suitable for these studies in the following ways: (1) it grows on a minimal medium that contains as organic components only a carbohydrate and biotin, (2) its developmental cycle requires only 3–4 weeks, and (3) it is haploid and allows the analysis of ordered tetrads.

1. Production of Mutants with Specific Deficiencies in Synthetic Steps

For the induction of mutations with nutritional deficiencies, the following methods were used (Beadle and Tatum, 1941; concerning methodology, see Ryan, 1950).

Conidia were irradiated either in the dried condition with X-rays or neutrons (doses up to 100,000 r.) or in water suspension with ultraviolet (doses up to 15,000 ergs/mm.^{2} at 2537 A.). Following irradiation, protoperithecia of the opposite mating type were fertilized with these conidia. From individual perithecia that were produced, single ascospores were isolated and brought to germination on complete medium. The complete medium, which contained yeast extract and casein hydrolyzate, thus provided vitamins, amino acids, purines, pyrimidines, and other growth factors and thereby permitted compensation for any nutritional defects brought about through mutational events. Since the mutative effect is transmitted in meiosis, the mycelium derived from an individual ascospore is homogeneous; all homosporous strains, which grew upon complete medium, were then tested on minimal medium. If no growth followed in this second test, it was assumed that the strain no longer was able to carry through all synthetic functions of the prototrophic wild-type strain. For the identification of specific deficiencies, these auxotrophic strains were then tested in a systematic test series on minimal media to which were added vitamins, amino acids, purines, pyrimidines, etc., individually or in combination. In this way it was possible to identify specific nutritional requirements of the auxotrophic strains, e.g., if a strain could grow only upon minimal medium supplemented with tryptophan, it could be designated as an auxotrophic mutant with a defect in its competence to synthesize tryptophan.

Through backcrosses of the mutants with wild type and an analysis of the progeny by tetrad analysis, the kind of mutation could be established. In the cases investigated to date, it has been shown that each of most mutants differs from wild type by a single altered gene. In this way, several hundred auxotrophic mutations have been induced

which possessed nutritional defects for vitamins (Horowitz *et al.*, 1945), for almost the entire known list of amino acids (Horowitz *et al.*, 1945), for purines, and for pyrimidines (Horowitz *et al.*, 1945; Loring and Pierce, 1944; Tatum, 1944). For certain of these substances, several non-allelic auxotrophs have been found.

Certain precautions need to be taken in the use of this method for the induction of mutations. For example, some mutants can grow only at a given pH (Stokes *et al.*, 1943), and if this pH is not used in the test, such mutations are not obtained. Also, multifunction genes cannot be detected in most cases (Atwood and Mukai, 1953).

A method used for several years for the procurement of auxotrophs for specific defects depends entirely upon selection (Woodward *et al.*, 1954); conidia, treated with mutagenic agents, are first brought to germination on a minimal medium. The ungerminated conidia are separated from the germinated conidia by filtration, and the ungerminated conidia are then plated selectively on minimal medium that contains the supplement corresponding to the auxotrophic mutants that are sought.

Auxotrophic mutations have been found meanwhile in a series of other fungi: *Ophiostoma* (Fries, 1947; Fries and Kihlman, 1948), *Ustilago* (Perkins, 1949; Holliday, 1961a), *Saccharomyces cerevisiae* (Lindegren, 1949; Lindegren and Lindegren, 1947; Pomper and Burkholder, 1949), *Aspergillus* (Pontecorvo, 1953); *Glomerella cingulata* (Markert, 1952; Wheeler, 1956a); *Sordaria fimicola* (El-Ani *et al.*, 1961); *Schizophyllum commune* (Raper and Miles, 1958; Ellingboe and Raper, 1962), *Coprinus lagopus* (Lewis, 1961a; Day, 1960a, 1961).

2. *Gene-Determined Biochemical Synthesis*

Proceeding from the fact that an organism requires a series of steps for the synthesis of a vitamin or an amino acid, the roles of different auxotrophs with the same nutritional requirement were solved through substitution for the specific need, e.g., arginineless mutants were tested with ornithine and citrulline (Srb and Horowitz, 1944), the amino acids previously known to be precursors of arginine in other organisms. Of 7 arginine-requiring mutations, 4 grew after supplementation with ornithine or citrulline; 2 grew after supplementation with citrulline but not with ornithine; 1 mutant responded neither to citrulline nor to ornithine but grew only with arginine. The logical significance of these results was that the different genes responsible for the character *arginineless* block the normal synthesis of arginine at different places. Thus, the wild alleles of these genes are required for individual steps in the synthetic

sequence in the order, ornithine–citrulline–arginine. Each of the individual steps which suffered a mutative accident can be compensated for by the addition of the corresponding product.

Through the use of this technique, auxotrophs of *Neurospora* have been used to achieve a more or less full understanding of a number of biochemical syntheses (Table II).

TABLE II

SYNTHETIC SEQUENCES PARTIALLY OR ENTIRELY ESTABLISHED THROUGH THE USE OF AUXOTROPHIC MUTANTS OF *Neurospora*[a]

Product	Literature
Proline and arginine	Srb and Horowitz (1944), Srb (1946)
Tryptophan and nicotinic acid	Tatum and Bonner (1943, 1944), Tatum *et al.* (1944), Beadle *et al.* (1947), Mitchell and Nyc (1948), Bonner (1948), Nyc *et al.* (1949)
Threonine and methionine	Horowitz *et al.* (1946), McElroy *et al.* (1947), Horowitz (1947), Teas *et al.* (1948)
Lysine	Doerman (1944, 1946), Houlahan and Mitchell (1948)
Isoleucine and valine	Bonner *et al.* (1943), Bonner (1946b)
Choline	Horowitz (1946), Horowitz *et al.* (1945), Jukes and Dornbush (1945)
p-Aminobenzoic acid and sulfanilamide	Tatum and Beadle (1942a), Emerson and Cushing (1946), Emerson (1947, 1948), Zalokar (1948)
Thiamine	Tatum and Bell (1946)
Riboflavine	Mitchell and Houlahan (1946b)
Pantothenic acid	Tatum and Beadle (1945), Wagner and Guirard (1948), Wagner (1949)
Succinic acid	Lewis (1948)
Purines	Mitchell and Houlahan (1946a), Pierce and Loring (1945), McElroy and Mitchell (1946), Loring and Fairley (1948)
Pyrimidines	Loring and Pierce (1944), Mitchell and Houlahan (1947), Mitchell *et al.* (1948)

[a] After Horowitz (1950).

Proof of the correctness of the concept that individual steps in biochemical synthesis are controlled *in vivo* by individual genes could, in certain cases, be confirmed by the identification of precursors. Precursors of vitamins or amino acids are quickly used in the normal organism, and their quantities are too small to be detected. If a synthetic step is blocked by a gene mutation, however, these precursors accumulate

in the nutrient medium and thereby provide important evidence of the course of the biosynthesis. It was thus found, through the accumulation of precursors, that the formation of homocysteine proceeds from cysteine by way of cystathione (Horowitz, 1947).

3. *The One-Gene-One-Enzyme Hypothesis*

The analysis of single-gene auxotrophic mutations of *Neurospora* and their "position" in specific biochemical reaction-steps has frequently shown that the specific effect of a mutation is to block a specific synthetic step. That enzymes in general are responsible for biochemical reactions has led to the hypothesis "that a large class of genes exists in which each gene controls the synthesis or the activity of but a single enzyme" (Horowitz, 1950). This concept has become known as the "one-gene-one-enzyme hypothesis" (Beadle, 1945a, b; cf. also Haldane, 1942; Wright, 1941). This hypothesis has proved to be a valuable working hypothesis for the clarification of most findings in *Neurospora*, and it has also called attention to the importance of biogenetic experimentation in other materials (literature: Horowitz, 1950). Of course, objections to the one-gene-one-enzyme hypothesis have been provided by further experimental work: e.g., Landman (1951) pointed out the probability both (*a*) that one gene may determine several enzymes and (*b*) that one enzyme may be influenced by several genes (cf. also Lederberg *et al.*, 1951). Another counterargument that is often raised is the fact that the auxotrophic mutants are secured through selection (Atwood and Mukai, 1953). Several findings of the most recent gene-enzyme research of numerous authors support the one-gene-one-enzyme hypothesis. More than 200 independent tryptophan-auxotrophic mutations obtained through selection all lie in a linear order in a single gene locus (Bonner *et al.*, 1960), and the same holds for 35 mutants with an adenylosuccinase defect (Giles *et al.*, 1957b; Giles, 1958) as well as for different mutations with an arginine-suceinase defect (Newmeyer, 1957). Further support for the hypothesis has been provided through the study of the enzyme, tyrosinase. The synthesis of tyrosinase is influenced by three different genes. Only one of these genes, however, influences enzyme structure; the other two control only the formation of enzyme depending upon the nutritional conditions (Horowitz *et al.*, 1960, 1961).

Further study (next section) concerned with the interdependence between gene and enzyme structure may eventually reveal whether and to what extent the one-gene-one-enzyme hypothesis must be considered as relevant.

C. Gene-Enzyme Relationships

1. Effect of Gene Mutations on Enzymes

The one-gene-one-enzyme hypothesis has led to the general assumption that a gene determines a specific function of an enzyme. Mutative change within a gene must then be expressed as a change or a loss of enzyme function. Since the end of the 1940's, a number of groups of workers have attempted, with auxotrophic mutations, to determine the enzymes responsible for specific synthetic steps and to determine the effect of mutation on the properties of the enzymes. As materials for these studies, both fungi and bacteria have been used (literature reviews: Fincham, 1959a, 1960; Bonner, 1959; Catcheside, 1960; Yanofsky, 1960; Yanofsky and St. Lawrence, 1960).

The most drastic effect of a gene mutation is the absence of enzyme formation in the mutant (Table III). In many cases, however, the

TABLE III
KNOWN CASES AMONG FUNGI OF CORRELATION BETWEEN GENE MUTATIONS AND ENZYME SPECIFICITY[a]

Enzyme	Enzymatic alteration in mutants	References
Saccharomyces cerevisiae		
Diphosphothiamine phosphatase	Increase	Tavlitzki (1954)
Galactokinase	Loss	de Robichon-Szulmajester (1958)
Glomerella cingulata		
Tyrosinase	Reduction in activity	Markert (1950), Markert and Owen (1954)
Podospora anserina		
Laccase	Qualitative alteration	K. Esser (unpubl.)
Neurospora crassa		
Locus / Enzyme		
ad-4 Adenylosuccinate synthetase	Qualitative alteration	Giles *et al.* (1957a)
aun Glutaminic acid dehydrogenase	Loss, qualitative alteration	Fincham (1954, 1957), Pateman and Fincham (1958), Fincham and Pateman (1957)
arg-1 Argininosuccinate synthetase	Loss	Newmeyer (1957)

[a] Adapted from Fincham (1959a) and Catcheside (1960).

TABLE III (*Continued*)

Enzyme		Enzymatic alteration in mutants	References
Neurospora crassa (*Cont.*)			
Locus	Enzyme		
arg-10	Argininosuceinase	Loss	Fincham and Boylen (1957)
car	Pyruvie carboxylase	Reduction in activity	Strauss (1953)
his-1	Imidazole-glycerol phosphate dehydrase	Loss	Ames (1957a)
his-5	Glutamate-phosphohistidinol transaminase	Loss	Ames and Horecker (1956)
his-3	Histidinol dehydrogenase	Loss	Ames (1957b)
his-4	L-Histidinol phosphate phosphatase	Loss	Ames (1957b)
IV-1	Dihydroxyisovalerate dehydrase	Reduction in activity	Myers and Adelberg (1954)
	Laetase	Reduction in aetivity	Landman (1950)
me-2	Cystathionase II,	Loss, reduetion in	Fischer (1957)
me-7	cystathionase I	aetivity	
	Nitrate reductase, nitrite reduetase	Loss	Silver and McElroy (1954), McElroy and Spencer (1955)
pro	Pyrroline-5-earboxalate reduetase	Qualitative alteration	Yura (1959)
pyr-3	Asparatie aeid earbamyl transferase	Loss	Davis (1960)
suc	Oxalaeetie acid carboxylase	Reduetion in activity	Strauss (1957)
td	Tryptophan synthetase tryp-3 (tr.-desmolase)	Loss, qualitative alterations	Yanofsky (1952), Yanofsky and Bonner (1955a), Suskind *et al.* (1955), Suskind and Kurek (1957), Mohler and Suskind (1960), DeMoss and Bonner (1959), Esser *et al.* (1960)
T *ty-1* *ty-2*	Tyrosinase	Qualitative alteration, loss	Horowitz and Fling (1953, 1956), Horowitz *et al.* (1960, 1961)

enzyme molecules are not completely absent; they may be present in an activatable form (Fincham, 1957) or in an inactive form as a serologically active protein, "cross-reacting material" or CRM (Suskind *et al.*, 1955; Suskind, 1957).

In other cases, the effect of a mutation may consist in a quantitative change of the enzyme which is shown primarily as a decrease of activity and only seldom as an increase. For the demonstration of quantitative differences of enzyme activity, it is necessary to observe that the same system (e.g., specific activity) is used for the comparison of enzyme activity of mutant and wild-type strains, that the same cultures always be employed and that the same conditions of enzymatic preparation be used for both strains. It must further be certain that there is not included in the preparation an inhibitor unrelated to the mutation that decreases the activity of the enzyme and thereby introduces a quantitative effect.

A further, deeper insight into the functional interdependence between gene and enzyme has been demonstrated in cases in which qualitative changes of enzymes have been brought about by mutation (Table III). The demonstration of a qualitative change in enzyme activity requires the application of complete knowledge of the properties of the wild enzyme; e.g., the following criteria can be applied: substrate specificity; sensitivity to inhibitors; stability against pH, heat, and oxidation; behavior in electrical and gravitational fields; molecular weight. For the determination of these criteria, extensive purification of the enzyme is necessary in most cases. The individual properties of the enzyme, however, are not changed in purification. It must further be certain that the qualitative change in the enzyme is not brought about by another cellular component associated with the enzyme. It is very probable that in certain of the cases given in Table III, quantitative differences (decrease, loss, or increase of enzyme activity) would have been shown by more extensive investigations to be qualitative differences.

Tyrosinase of *Neurospora* is an example of an enzyme for which a known gene exerts a qualitative as well as quantitative effect on enzymatic activity. Tyrosinase catalyzes the oxidation of mono- and dihydroxyphenol compounds and is thereby involved in the synthesis of melanin pigment. Horowitz *et al.* (1960, 1961) have been able to show that the locus *T* is responsible for the structure of this enzyme. Allelic mutations at this locus cause a qualitatively changed tyrosinase, i.e., changed heat stability and different electrophoretic behavior. In addition to the *T* locus, two other loci, *ty-a* and *ty-2*, unlinked to *T*, are known to exert a quantitative influence on enzyme formation.

There are as yet no experimentally corroborative data from *Neurospora* concerning the place and manner in which the structure of enzymes is qualitatively changed through mutation. In the bacterium *Escherichia coli*, however, altered peptide components of the A protein of tryptophan synthetase have been correlated with mutations (Yanofsky *et al.*, 1961). It is assumed that through mutation, a change in amino acids sequence results and that the catalytically active centers are changed or masked through alterations in tertiary structures (e.g., folding).

In addition to the direct effects of mutated genes on enzyme structure, several cases are known in which mutatively changed genes have an indirect influence on particular enzymes. Such effects may involve the induction of inhibitors (Silver and McElroy, 1954) or a change of the required nutrients (literature: Fincham, 1960).

2. *Fine Structure of the Gene and Enzyme Specificity*

The facts that the formation of an enzyme is controlled by a gene and that mutative changes within a gene locus can lead to quantitative alterations of the enzymes have led to the further broad assumption that the base sequence of DNA, as postulated in the Watson-Crick model, serves as a code to specify the amino acid sequence of an enzyme. For this assumption there is to date no good experimental evidence. To this end, the goal of modern biochemical genetics is to obtain evidence concerning the base sequence of a gene and the sequence of amino acids of the corresponding enzyme. One approach to this goal lies in the comparative genetic analysis of the fine structure of a gene parallelled by a biochemical analysis of the enzyme determined by this gene. Such studies have been undertaken by several groups of workers with *Neurospora* and bacteria (literature: Bonner *et al.*, 1960; Fincham, 1960; Yanofsky, 1960; Yanofsky and St. Lawrence, 1960). As a representative example, we will describe in the following the investigations of Bonner and associates on the *td* locus and the enzyme tryptophan synthetase controlled by this gene (for literature, see Bonner *et al.*, 1960; Yanofsky, 1960).

Tryptophan synthetase catalyzes the last step in the synthesis of tryptophan: the substitution of serine for glycerol phosphate in indoleglycerol phosphate. This conversion (reaction 1), requires pyridoxal phosphate as a cofactor (Umbreit *et al.*, 1946). In addition, tryptophan synthetase also catalyzes two further reactions: the coupling of indole and serine to form tryptophan (reaction 2) and the transformation of indoleglycerol phosphate to indole (reaction 3). Through systematic selective methods, somewhat more than 200 mutations (mostly UV) for

the *td* locus were obtained. All these mutations were auxotrophic for tryptophan and incapable of carrying out reaction 1. A biochemical analysis showed functional differences between these mutations (Table IV).

TABLE IV

FUNCTIONAL DIFFERENCES FOUND AMONG MUTANTS AT THE *td* LOCUS IN *Neurospora crassa*[a]

Group I:	Enzyme activity lost
II:	Formation of CRM, a protein serologically related to tryptophan synthetase.
Class 1:	Inactive for reactions 1, 2, 3
2:	Inactive for reactions 1 and 3; active for reaction 2
3:	Inactive for reactions 1 and 2; active for reaction 3

	Cofactor for class 3 mutants	
Subclass	Pyridoxalphosphate	Serine
a	—	—
b	+	—
c	+	+

[a] Adapted from Bonner *et al.* (1960).

A further classification of the mutants can be made on the basis of temperature sensitivity, sensitivity to zinc ion, and effect of suppressors.

These findings thus show that the *td* locus is complex and may contain many mutational sites. This conclusion was confirmed by analysis of the fine structure of the *td* locus by means of crosses of the mutants with one another: each cross between two mutants yielded a characteristic frequency of prototrophs [see sections on Factor Analysis (V, A), Gene Conversion (V, A, 6), and Gene Mapping (V, A, 4)]. From the

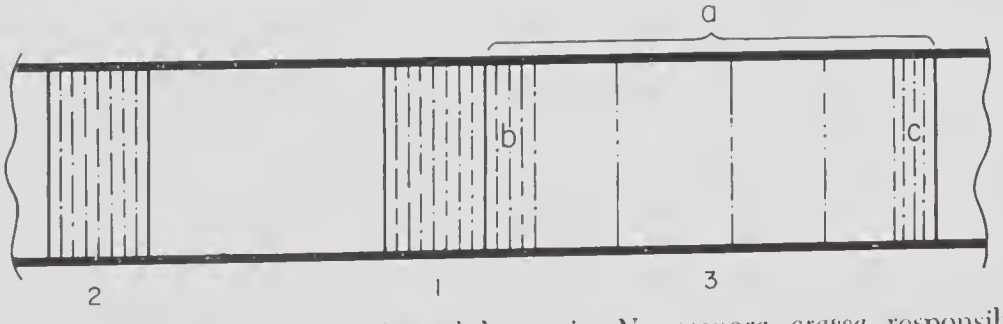

FIG. 9. Schematic map of the *td* locus in *Neurospora crassa* responsible for the formation of tryptophan synthetase. Only the mutants from group II (Table IV) are shown. The classification of the different regions of the *td* locus is also taken from Table IV. With the exception of the mutants of subclass 3a, which are scattered within class 3, the different mutant types are localized in distinct regions of the *td* locus. No mutants have been found to lie in the region between classes 2 and 1. Adapted from Bonner *et al.* (1960).

frequency of prototrophs it was possible to order linearly the individual mutants within the *td* locus. Surprisingly, the individual mutants were not randomly scattered over the entire region, but revealed a correlation between localization and function (Fig. 9).

The mutations of group I are not ordered, and these probably involve deletions or multisite mutations rather than single-site mutations.

These functional subdivisions of the *td* gene provide the basis for further genetic and biochemical investigations to seek evidence of the mechanism by which DNA exerts its physiological activity.

3. *Intragenic (=Interallelic) Complementation*

Complementation of the corresponding wild alleles between nonallelic auxotrophs to restore nutritional competence is a well-known phenomenon of widespread occurrence. In fungi, as in bacteria, it has been inci-

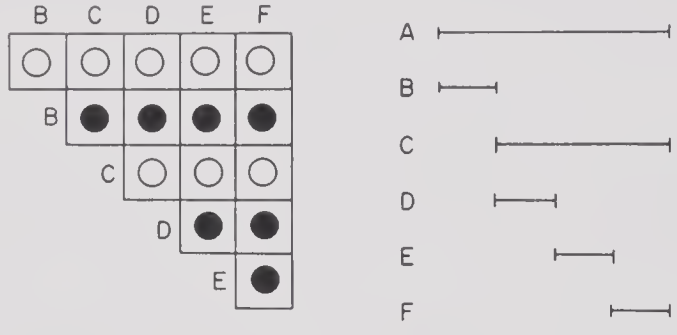

FIG. 10. Complementation relationships between, and complementation map of, *his*-3 mutants in *Neurospora crassa*. The filled circles represent complementation; the open circles, the absence of complementation. The basic assumption for the construction of the linear complementation map (right) is that complementation occurs only in nonoverlapping regions. From Catcheside (1960).

dentally observed that all auxotrophs can complement and in this way lead to prototrophic growth. Complementation occurs either in heterozygotes (in yeasts, Roman, 1956), in heterokaryons (in fungi, Fincham and Pateman, 1957; Giles *et al.*, 1957b), or through abortive transduction (in bacteria, Hartman *et al.*, 1958).

On the basis of complementation, auxotrophic mutations of a locus can be subdivided into groups. Within each group no complementation occurs, and, depending on whether complementation occurs between the different groups, they can be linearly ordered. In this way a gene locus can be charted by the functional properties of individual mutations (Fig. 10). The adequacy of this method follows from the near to complete agreement of the complementation maps for the *td* locus of *Neurospora*, independently constructed by Ahmad and Catcheside (1960) and by Lacy and Bonner (1958, 1961).

Additional complementation maps have been made for different loci in *Neurospora crassa* (Catcheside and Overton, 1958; Case and Giles, 1958b, 1960; Partridge, 1960; Woodward *et al.*, 1958; Murray, 1960; Wagner *et al.*, 1960; de Serres, 1960).

The complete agreement of complementation maps with the gene maps constructed from conversion data is as yet unproved. Case and Giles (1958b, 1960) found only a general agreement in the corresponding sequences of individual mutant types. Of particular interest is the observation of restored enzymatic activity through intragenic complementation. Such heterokaryons, which never approached the growth of prototrophs, produced, as compared to wild type, quantitatively less enzyme (Catcheside, 1960; Fincham, 1959a) as well as partially qualitatively altered enzyme (Fincham, 1959b; Partridge, 1960). Of special significance in this connection are the investigations of Woodward (1959), who was able to demonstrate complementation *in vitro* between extracts of allelic adenylsuccinase auxotrophs of *Neurospora crassa*.

4. Actions of Suppressors on Enzymes

Through suppressor mutation, the activity of another, and usually nonallelic, mutation is entirely or partially restored. Suppressor genes have often been described for auxotrophic mutations of fungi (Giles and Partridge, 1953; Strauss and Pierog, 1954; Lewis, 1961a; etc.). Biochemical investigations concerning the mode of action of suppressor mutations, however, have as yet been made only for the *td* locus of *Neurospora crassa* and the bacterium *Escherichia coli* (Yanofsky, 1952, 1958; Yanofsky and Bonner, 1955b; Yanofsky and Crawford, 1959; Suskind and Kurek, 1957, 1959; Suskind and Jordan, 1959).

For tryptophan synthetase mutations of *Neurospora crassa*, the wild character could be partially restored through suppressor genes; i.e., prototrophy is only partially restored. In no cases have the suppressed mutants given the same growth rate or the same enzyme production as the wild strain. The different suppressor genes possess a specific activity on individual *td* alleles, e.g., suppressor-2 suppresses only *td*-2 but none of the other 24 confirmed and nonidentical mutant *td* alleles. The specific suppressor genes for the different *td* alleles were, in general, located in different portions of the genome. It is interesting that for the mutant *td*-1 (Group I, Table IV, no CRM), no suppressor genes have been found. This result leads to the hypothesis that the suppressor gene, as a substitute for the *td* locus, cannot function to provide the code for enzyme formation, but rather that the suppressor has only modified the molecule built by the *td* locus, i.e., CRM or inactive enzyme. This influence can either

originate in an alteration of the intracellular conditions so that the formation of the normal enzyme is restored or an alteration in the secondary or tertiary structure of the enzyme is induced. Further experimental findings concerning suppressor genes give no evidence against an indirect participation of suppressor loci in enzyme synthesis.

5. Action of Reverse Mutations on Enzymes

Reverse or back mutations which restore the wild phenotype have frequently been described in microorganisms. Since it is known that all alleles are located in different sites within a genetic region, the question rises whether the reverse mutation, e.g., auxotrophy to prototrophy, produces not only a phenotypic wild type but also, at the molecular level, the original wild type. This problem has been investigated by many authors with *Neurospora crassa* (Pateman, 1957; Giles, 1956, 1958; Fincham, 1957; Woodward *et al.*, 1958; Woodward, 1960; Esser *et al.*, 1960) and with *E. coli* (Maas and Davis, 1952; Yanofsky and Crawford, 1959; Stadler and Yanofsky, 1959; Garen, 1960).

Giles was able to show, in his investigation of the *ad*-4 locus in *Neurospora crassa*, that the revertants of different classes having quantitative enzyme differences could probably be achieved through qualitative, that is, structural, enzyme differences. Yanofsky and co-workers reached the same conclusion in their studies on *E. coli*.

An adequate conception of the biochemical properties of reverse mutations is now available for the *td* locus of *Neurospora crassa* (Esser *et al.*, 1960). Among 25 revertants of the mutant *td*, at least four different classes can be distinguished in respect to growth rate and morphology as compared with wild type. The individual classes of revertants could be differentiated, one from another, by qualitative enzyme differences. The revertants of the first class, which constituted half of all the mutants analyzed, produced tryptophan synthetase that could not be differentiated from that of wild strains; representatives of the other classes, without exception, formed enzymes different from wild type. The authors assumed that, at least in these mutants, the last three classes do not represent single point reversions, but that the conversion to prototrophy through mutations can be effected in other sites of the *td* locus. The concept of "intragenic suppression" could be confirmed by further experimental work.

When the relationship between genes and enzymes is examined in the light of all experimental findings, it can only be recognized that our information at the moment is still too fragmentary to permit the formulation of a general theory. The way in which progress can be made,

however, is clear. Biochemical analysis of the fine structure of enzymes must proceed parallel to genetic investigations until a complete correlation between the two is possible through the resolution of the genetic code.

Such reflections prompt the question of whether, and to what extent, the one-gene-one-enzyme hypothesis is applicable at the present time. Actually to date, very few experimental results argue against this hypothesis. The case is exactly the opposite: the extensive investigations of complex loci, the individual mutated sites of which are able to form structurally different enzymes, speak for the hypothesis that considers the gene a physiological unit. For, in spite of all enzymatic and genetic differences, the gene can be considered only in relation to an enzyme with a given biocatalytic function.

The one-gene-one-enzyme hypothesis requires, of course, a formulation adjusted to new information, which, however, changes in no way the meaning of the hypothesis. Definite segments of genetic material, constituted of DNA, are responsible for the specificity of polypeptides. These polypeptide chains are linked together to form protein, which can be physiologically active. A series of such segments, responsible for the formation of such a protein, forms a physiological unit corresponding to the earlier gene. Mutation within the individual DNA segments brings about a change in the DNA code, which is reflected in a change of the polypeptide specificity and is manifested finally as a change in the structure of the protein and consequently its activity, especially the specificity of an enzyme.

D. Extrachromosomal Inheritance

Carl Correns, one of the rediscoverers of Mendel's laws, recognized in 1909 that the cytoplasm of the cell as well as the nucleus carried heritable characters. Since that time, many such extrachromosomal phenomena of inheritance have been described in plants and animals, and it has become clear that these heritable characteristics located outside the chromosomes may possess the competence for self-duplication as well as that of inheritance by sexual or asexual means. The expression of phenomena conditioned through extrachromosomal characters is, in many cases, completely dependent on the genome. The determination of extrachromosomal inheritance can have its focus either in the plastids or in the remaining portions of the cytoplasm. By analogy to the expression of the genome, which includes the sum of the hereditary material lying in the chromosomes, all extrachromosomal factors of inheritance are classified as *plastom* (heritable characters of the plastids) and *plasmon*

(heritable characters in the rest of the cytoplasm). Since the fungi possess no plastids, we will in the following be concerned only with the plasmon.

An essential characteristic of extrachromosomal inheritance finds its expression in the fact that among the progeny of a cross, the partners of which possess a difference of plastom or plasmon, the sort of segregation typical for chromosomally inherited properties has never been found. The charaeteristic transmission of extrachromosomally inherited traits is frequently termed "maternal inheritance." This follows from a characteristic of fertilization in most organisms. In most plants and animals, the cytoplasmic portion of the fertilized egg cell is predominantly contributed by the female; the male partner contributes only little, or essentialy no, cytoplasm. This is also the case in the Euascomycetes and the rust fungi, whereas in the Hymenomycetes and smut fungi, it is unknown whether cytoplasmie transfer occurs in connection with nuclear transfer. Maternal inheritance leads to reciprocal differences in the progenies of crosses in haploid as well as diploid organisms.

Nevertheless, when the cytoplasm of the two fusing cells contribute equally in the formation of the zygote e.g., in yeast, no segregation occurs among the progeny in crosses that are heterogeneous for extrachromosomally inherited characters. The different cytoplasms of both parents become mixed in the course of the formation of the zygote and are passed along to the progeny as a mixture; all of the progeny receive the same mixture and are thereby uniform. In haploids, the failure of segregation can be recognized in the F_1, whereas in diploids, it can first be recognized in the F_2.

The Mendelian laws thus have no application in extrachromosomally determined hereditary phenomena. As a result of the failure of segregation, the plasmon was for a long time considered as an undivided whole, since definitive components of the plasmon could not be demonstrated as could the genes of the genome. Many cases of extrachromosomal inheritanee were early known and more were occasionally found, but they tended to remain in an almost mystical darkness in respect to their eomponents. One of the objectives of recent research has thus been to distinguish between genes located in the chromosomes and the determinants of extraehromosomal inheritance. Only in the last two decades has it been possible to relate, with any certainty, structures located in the cytoplasm with extrachromosomal inheritance. In these researches the fungi have played a significant role as experimental materials. Numerous comprehensive accounts deal with extrachromosomal inheritance—*general*: Correns (1937), Caspari (1948), Ephrussi

(1951, 1953), Oehlkers (1952), Rhoades (1955), Lindegren (1957), Nanney (1957), Mather (1948), Catcheside (1959); *special (other than fungi)*: Michaelis (1951), L'Héritier (1951), Beale (1954), and Sonneborn (1959); *special (fungi)*: Ephrussi and Hottinguer (1951), Ephrussi (1952, 1956), Srb (1958), Windisch (1958).

1. Mitochondria as Extrachromosomal Hereditary Carriers

Ephrussi and co-workers (Ephrussi, 1956; Ephrussi *et al.*, 1949a, b, c; Tavlitzki, 1954; Slonimski, 1949a; Slonimski and Ephrussi, 1949; Ephrussi and Slonimski, 1955) have shown that, in the course of growth of haploid or diploid cultures of bakers' yeast, *Saccharomyces cerevisiae*, on a solid nutrient medium, small, "petite," colonies occurred with a frequency of approximately 1%. The cells of these small colonies can be isolated and grown in pure culture. The small size of the colony is determined by the inability to respire glucose and a consequent depressed rate of cellular division as compared to that of normal yeast cells. Yeasts in general can obtain their energy requirements either through respiration or through fermentation of carbohydrates. If the respiration of yeast cells is suppressed through endogenous or exogenous factors, they can still propagate vegetatively by fermentation, although their division rate is essentially depressed proportionately to the decreased energy source. There are no morphological differences between cells of the small colonies and normal cells.

The respiratory defect of the cells of the small colonies is a "vegetative mutation" and results from an altered condition of the respiratory enzymes. In the vegetative mutations, at least four known enzyme systems are completely lacking: cytochrome oxidase, succinocytochrome-c reductase, DPNH-cytochrome-c reductase, and α-glycerolphosphate dehydrogenase. Cytochromes a and b are also defective. Other smaller but decisive enzymatic changes accompany these defects (Slonimski, 1949a, 1950, 1952; Slonimski and Ephrussi, 1950).

If the vegetative mutants are crossed with wild type, the character "small colony = respiration defect" is lost in the progeny. It appears neither in the diploid generation, nor among the haploid progeny of the asci, with the exception of the usual 1%. This loss of expression clearly means that the respiratory defect cannot be determined by genes of the nucleus and that its locus of effect must be sought outside the chromosomes.

The frequency of mutation from wild to *petite* can be significantly increased by treatment of the cells with chemicals (acridine dye, tetra-

zolium chloride) or changes of physical conditions, e.g., ultraviolet light, heat (literature: Windisch, 1958). Spontaneous and induced mutations agree in their biochemical properties. They show differences in the genetic behavior, however, which lead to a classification of these mutant types into (*a*) neutral, i.e., those previously mentioned, and (*b*) suppressive vegetative mutants (Ephrussi *et al.*, 1954, 1955). As compared to the neutral mutations, the suppressive mutations behave exactly opposite in crosses with wild type: their character determines the total progeny, which thus consist only of respiration-defective cells. Furthermore, the suppressive mutations mutate after brief vegetative propagation to neutral. Mutation from neutral to suppressive has not yet been observed.

To answer the question, whether definite particles of the cytoplasm are responsible for the respiratory defects of vegetative mutants and thereby to designate them as extrachromosomal heritable traits, the following observations are relevant. (1) The four respiratory enzyme systems missing from the vegetative mutants are normally bound to the centrifugable grana of the cytoplasm (Slonimski and Hirsch, 1952). (2) Through cytochemical methods, it is possible to demonstrate that the mitochondria are the site of cytochrome oxidase. (3) In the respiration-defective mutants, little cytochrome oxidase, among others, is produced. Mitochondria, however, are formed (Yotsuyanagi, 1955). These observations and others not discussed here in detail have led Ephrussi and his co-workers to the conclusion that with a high degree of probability the mitochondria can be considered as the carriers of extrachromosomal hereditary characters.

Vegetative mutations differ, therefore, from the wild type through the possession of inactive mitochondria which are not capable of synthesizing the respiratory enzymes. In a cross between mutant and wild, active and inactive mitochondria are mixed, and the cell is capable of respiration. The mitochondria are also capable of self-duplicating, and all daughter cells contain sufficient active mitochondria. Inactive mitochondria can originate from active through spontaneous, irreversible mutations, and the spontaneous appearance of vegetative mutations may be considered as due to the chance inclusion of inactive mitochondria in budding daughter cells.

Inactive mitochondria of the vegetative mutants of the suppressive type, however, inhibit the function of otherwise capable mitochondria of wild type. As a result of their high frequency of mutation to neutral type, this effect is lost after several cell generations.

An extremely important relationship between extrachromosomal and

chromosomal hereditary factors for respiration has been revealed in the discovery and genetic analysis of another small-colony mutation. The respiratory defect of this mutation is due to the same enzymatic deficiencies found in vegetative mutation, but this defect is determined by a nuclear gene. In crosses with the wild type, this mutant always yields the expected 2:2 segregation in the asci. Diploid cells derived from crosses with wild type were normal. Furthermore, crosses of the segregational mutant with vegetative mutants yielded normal diploid cells, and, in the ascus, yielded 2:2 segregation for normal and defective. The segregational mutant obviously carries a recessive gene, which inactivates its normal mitochondria or inhibits their activities. The vegetative mutant possesses, on the contrary, the normal dominant allele of this gene but no functional mitochondria. The normal growth of the diploid cells, which originate from zygotes of both mutants, is therefore the consequence of a mixing of physiologically inactive but potentially normal mitochondria from the segregational mutant with the dominant allele of the nucleus of the vegetational mutant. The inactive particles regain their full activity in this way in the formation of the ascus and are distributed to all four spores; only half of the spores, however, carry dominant alleles following segregation from the heterozygous diploid nucleus.

These experiments show (1) that the formation of respiratory enzymes is determined by an interaction between chromosomal and extrachromosomal heritable factors and (2) that the extrachromosomal heritable factors are, in this case, probably distinct, self-duplicating particles of the cytoplasm, the mitochondria.

Extrachromosomal genetic phenomena associated with the mitochondria also seem to occur in *Neurospora crassa* in a mutant that has become known as *poky* (Mitchell and Mitchell, 1952; Mitchell *et al.*, 1953; Haskins *et al.*, 1953). A nuclear-cytoplasmic relationship has not yet been demonstrated in this case. *Poky* mutations are distinguished, as the name well implies, by slow growth of the mycelia. This trait is inherited only maternally, i.e., all the spores from a protoperithecium of a *poky* race give rise to *poky* mycelia. Conversely, if *poky* conidia are used to fertilize wild-strain protoperitheeia, only wild mycelia are obtained from the ascospores. The *poky* character is not determined by an infectious particle, since it is not carried to the wild hyphae through anastomoses. Biochemically, the *poky* strain can be characterized by a changed complement of cytochromes and respiratory enzymes as compared with the wild type.

In *poky* cultures, only little cytochrome a and cytochrome b are

produced, which in older cultures reach only about 2% of the quantities in the wild strain. *Poky* produces, however, larger quantities of cytochrome c. An analysis of the particulate fraction of the cell-free enzyme extract, obtained through centrifugation, showed the absence of the respiratory enzymes succinic acid oxidase and cytochrome oxidase in the young *poky* cultures. With increasing age of the culture, however, succinic acid oxidase activity reaches the level of the wild type, while the activity of cytochrome oxidase remains subnormal. In the *poky* races, moreover, there can be demonstrated a cytochrome-decomposing enzyme, "cytochromase," that is absent in the wild strain. The higher cytochrome content of *poky* is possible only because still another system, at present inadequately characterized, is simultaneously produced that inhibits the activity of cytochromase.

Still a second extrachromosomally inherited mutant, *mi*-3, having similar growth properties and similar cytochrome content to those of *poky*, was isolated by the same authors. *Poky* and *mi*-3 differ both in their phenotypes and in their growth rates. This difference also occurs in the offspring of reciprocal crosses between *poky* and *mi*-3. Furthermore, there is no complementation between the two defective strains in the heterokaryon (Gowdridge, 1956). A cross between *poky* and a *mi*-3 with gene-determined growth mutations possessing similar properties (C-115 and C-117), revealed no correlation between characters inherited through the nucleus and characters transmitted by extrachromosomal means. It was concluded that cytoplasms of *poky* and *mi*-3 possess altered cytochrome systems that are transmitted from generation to generation without modification by nuclear genes. Since the cytochromes and the respiratory enzymes are associated with mitochondria, as shown above for the respiratory defective mutants of yeast, it can be assumed that *poky* as well as *mi*-3 mutations contain altered mitochondria. It can further be assumed that these mutated mitochondria possess genetic continuity, since they are in no way influenced by nuclear genes. It is very probable that the mitochondria are thus the carriers of these extrachromosomal hereditary phenomena in the same manner as in the small colonies of yeast.

2. *Infectious Particles of an Unknown Nature as Extrachromosomal Heredity Factors*

Rizet and his collaborators have been concerned, since the beginning of the 1950's, with the analysis of phenomena of extrachromosomal inheritance in *Podospora*, particularly *barrage* and *senescence* (Rizet, 1952, 1953a,b, 1957; Schecroun, 1958a,b, 1959; Rizet and Marcou, 1954;

Marcou, 1954a,b, 1957, 1958, 1962; Marcou and Schecroun, 1959; Beisson-Schecroun, 1962). Since the findings concerning barrage have earlier been reviewed (Ephrussi, 1953; Rhoades, 1955), we will first consider the lesser-known phenomenon of *senescence* which, in its genetic basis, is similar to that of the *barrage*.

a. Senescence. Wild strains of *Podospora anserina*, grown from ascospores, show the phenomenon of *senescence* after prolonged vegetative growth: their growth rate becomes slower, the thick cells lying back of the hyphal tip begin to die off, and the hyphal tips swell to form bubbles. The time of the beginning of *senescence* is specific for each wild strain. In the strain predominantly used for the first experiments, it began at 40 days at a culture temperature of 24 C. At higher temperatures, *senescence* begins earlier, at lower temperatures, later.

Senescent strains do not lose the competence, however, to produce sexual organs and fruit bodies. The progeny differ in reciprocal crosses between senescent strains and their still juvenile offspring, maintained at 4°C. Ascogonia of the juvenile strain, fertilized with spermatia of senescent strains, produce perithecia, the asci of which contain spores that germinate to normally growing mycelia. The reciprocal cross (senescent protoperithecia × juvenile spermatia) yields up to 90% senescent progeny. Thus all ascospores of a perithecium show the same phenotype, i.e., *senescence* is thus inherited in a purely maternal manner: the senescent status of a single nucleated female sexual organ, the ascogonium, determines the degree of *senescence* of all spores formed in the perithecium. The extrachromosomal inheritance of this character was thus demonstrated.

The results of microsurgical investigations on individual hyphae permitted formulation of a hypothesis concerning the nature of the hereditary characters responsible for *senescence*. When a juvenile hypha is placed against a senescent hypha, there occurs after a short time the formation of hyphal connections, or anastomoses. If the two hyphae are separated immediately after fusion, both maintain their original character. Following separation after some hours, however, the young hyphae have become senescent at the point of fusion and in the neighboring regions. The extent of *senescence* in the young hyphae depends upon the duration of contact in anastomosis. Since, in these experiments, both hyphae were marked with genetically different nuclei, nuclear migration could definitely be excluded. Diffusible substances were also excluded in further experiments. The transfer of *senescence* can occur only through hyphal contact, and it can be transferred in the same manner from the original juvenile hypha to another juvenile hypha.

From these and similar experiments, Rizet and co-workers concluded that *senescence* is determined by infectious particulate components of the cytoplasm. These particles, the nature of which is still unknown, must be self-duplicating, since they propagate in the cytoplasm of a juvenile strain when the particles are transferred via hyphal contact from a senescent strain. Since *senescence* occurs in every wild strain after a shorter or longer time, one must further assume that each strain must contain from the first, precursors or inactive proparticles, which achieve their infectious character only after protracted vegetative growth. These concepts can be made more precise by an examination of the following investigations on the barrage phenomenon.

b. The barrage phenomenon. Although no relationship could be found between the hereditary factors responsible for *senescence* and nuclear genes, a nuclear-cytoplasmic relationship occurs in the barrage phenomenon similar to that found in yeast for the respiratory character (see above). Rizet designated as *barrage* a 1 to 2 mm. pigment-free zone that was always formed between strains of different geographical races, regardless of their mating types. Grown upon certain media, the mycelia produced green-black melanin pigments. Pigments do not form in hyphal tips located in the barrage zone. Fruiting bodies are not formed within the barrage zone, but on both sides of this zone within pigmented regions of the mycelium. Barrage-formation thus normally does not inhibit sexual development. In most of the cases investigated, barrage-formation was released exclusively by nuclear genes (Esser, 1959a). One well-studied exception occurs between races *S* and *s*, and, in this, hereditary factors in the cytoplasm played an additional decisive role for the release of the phenomenon.

Races *S* and *s* differ by one gene. In the F_1 of $S \times s$, there occurs not the expected segregation of *S* and *s*, but of *S* and a modified *s*, which gives a barrage reaction neither with *S* nor with *s*. It has been demonstrated, by appropriate back crosses, that this modified-*s* race actually carries the unchanged gene *s*, and that its phenotype is determined by a cytoplasmic alteration. This cytoplasmic modification shows a typical maternal inheritance and remains constant through many sexual generations.

In longer vegetative propagation, the modified-*s* race has been observed, however, to revert to *s* with a frequency of about 10^{-7} per cell. This reversion spreads through the mycelium as an infection with a speed of about 7 cm. per day. The reversion can be effected by cytoplasmic contact with the original *s* races in anastomose formation by use of the same microsurgical techniques used in the *senescence* phe-

nomenon (see above). Also in this case, as in *senescence*, diffusible substances and nuclear migration can be excluded as responsible for the reversion, and infectious self-duplicating particles are implicated.

Rizet and co-workers give the following interpretation of the *barrage* phenomenon. The character *s* is determined by cytoplasmic particles which can be expressed only in the presence of the *s* nucleus. In the heterozygous ascus *S*/*s*, these particles are either eliminated or inactivated under the influence of *S* nuclei. The modified-*s* mycelia contain the *s* nucleus but no active particles. The reversion of modified-*s* races to normal races thus depends upon a spontaneous activation of previously inactive particles or upon the *de novo* occurrence of the particles. For the new origin of particles, a model hypothesis is suggested which corresponds to the findings concerning lysogeny in phage and episomes in bacteria (Jacob *et al.*, 1960). It is assumed that the *s* nucleus contains a proparticle, analogous to the prophage, that can change spontaneously in the cytoplasm and propagate itself further as an infectious particle. In the same way, the regular spontaneous occurrence of *senescence* can be explained through liberation of proparticles integrated in the nucleus. These investigations of *Podospora* have provided the information that infectious particles can frequently be responsible for phenomena of extrachromosomal inheritance, which may be expressed either independently of the genome, i.e., *senescence*, or only with a definite genome, e.g., *barrage*. The spontaneous occurrence of such particles further justifies the assumption that they can exist in two conditions, inactive and active, of which the latter is infectious and self-duplicating. Concerning the inactive form, nothing in the experimental literature contradicts their classification as proparticles corresponding in their behavior to prophages and episomes.

In the last few years, associates of Rizet and other authors, have reported several additional cases of extrachromosomal inheritance in the fungi that can be considered to be based upon infectious particles. Since these cases have not been analyzed in detail as have the two *Podospora* cases, we will only cite them here. These cases include the investigation of Sharpe (1958) on *Aspergillus glaucus*, Chevaugeon and Lefort (1960) and Chevaugeon and Digbeu (1960) on *Pestalozzia annulata*, and Cuzin (1961) on *Curvularia pallescens*.

In the investigation of phenomena of extrachromosomal inheritance in *Aspergillus glaucus* by Jinks (1954, 1956, 1957, 1958) and Roper (1958), a definite nuclear-cytoplasmic interdependence has been demonstrated. Mahony and Wilkie (1958) and Arlett (1960) also noted, on the basis of their studies on Aspergillaceae, cytoplasmic variability.

In these eases, however, extrachromosomal inheritance was not demonstrated with eertainty.

It can thus be said about the status of our knowledge of extrachromosomal inheritanee that this mode of inheritance is apparently widespread and, in most investigated eases, stands in a critical relationship with nuelear genes, whieh aet as a sort of eontrol center to determine the eompetenee to express extraehromosomally inherited eharaeters. About the particulate nature of the determinants of extrachromosomal inheritanee, no doubt ean remain in the three elassic cases of *petite*, *poky*, and *barrage* deseribed above. Although mitoehondria assume the role of determinants in the first two cases, the nature of the genctic earrier remains unknown for *barrage* and for the other phenomena of extraehromosomal inheritanee in the Ascomyeetes. Their infeetious eharacter, whieh is reminiseent of the known virus-like extrachromosomal heritable eharacters in *Paramecium* [kappa partieles (Sonneborn, 1959)] and in *Drosophila* [CO_2 sensitivity (L'Héritier, 1951)], permits a possible clarifieation eomparable to the lysogene- or episomemodels. These investigations of the fungi are important eontributions to our knowledge of the nature of extraehromosomal inheritanee in that they have provided definitive demonstrations of the earriers of the hereditary traits in these systems.

VI. Special Fungal Genetics

The eoncept of speeial genetie systems, either peculiar to the fungi or peeuliarly prominent in the fungi, had its origin in the work of Burgeff (1912, 1914, 1915) on sexuality and heterokaryosis in the Mucorales and in the independent discovery by Bensaude (1918) and Kniep (1918) of the relation of dikaryosis to sexuality in the Basidiomycetes. These early genetie studies have lead directly into two major problem areas that are eentral to the speeial biology of the fungi: incompatibility and heterokaryosis. (1) Previous work and work then in progress on sexuality in the Mueorales and Basidiomycetes formed the basis of Kniep's (1920, 1922) recognition of ineompatibility as a fundamental determinant of mating eompetenee in the fungi. (2) Subsequent work has revealed heterokaryosis to be very widespread among fungi and further to be a fungal monopoly. Together with the eorollary proeesses of parasexuality and somatic reeombination and the role of the dikaryon in the sexual eycle of the higher fungi, heterokaryosis must be recognized as the outstanding, unique biological feature of the fungi. To these three facets of fungal biology that deserve speeial genetic consideration here, ineompatibility, heterokaryosis, and parasexuality

and somatic recombination, a fourth, pathogenicity, should perhaps be added for completeness. Pathogenicity, however, lies somewhat peripheral to the scope of the present account and is the subject of numerous recent reviews (Yarwood, 1956; Barnett, 1959; Kiraly and Farkas, 1959; Day, 1960b).

A. *Genetics of Incompatibility*

Incompatibility, the genetic determination of mating competence in the absence of morphological differentiation, is known to occur in the Angiospermae (review: Lewis and Crowe, 1958) and in one species of the Filicinae (Wilkie, 1956) of the vascular plants and in the fungi. A catalog of the various systems of incompatibility in higher plants and in fungi is presented in a recent review by Esser (in press), who recognizes two major types of incompatibility: (1) *homogenic*, in which incompatibility results from like factors in the two mates, and (2) *heterogenic*, in which mating is barred by unlike factors.

1. *Homogenic Incompatibility*

Homogenic incompatibility systems of a variety of different types occur roughly in parallel in flowering plants and in fungi; common to both groups are systems based upon two factors of one series, multiple factors in one series, and multiple factors in each of two series. Two incompatibility systems, however, are each peculiar to only one of the two different groups: two factors in each of two series (tristyly in flowering plants) and two factors in one series and multiple factors in a second series (in certain smut fungi, Rowell and DeVay, 1954; Rowell, 1955).

The simpler of the incompatibility systems in the fungi, those having only two alternate factors and occurring in the Ascomycetes and the rusts and some smuts of the Basidiomycetes are currently the subject of little intensive study, and their characterization above is adequate for present purposes (Section III, B). The more complex systems, those having extensive series of alternate factors in one series or in two series and common among the higher Basidiomycetes, by contrast, are under vigorous investigation in numerous laboratories in several countries, and much significant information has become available since the publication of detailed reviews (Whitehouse, 1949b; Quintanilha and Pinto-Lopes, 1950; Raper, 1953; Lewis, 1954; Papazian, 1958). This applies in particular to tetrapolar incompatibility, and the following consideration is devoted to this complex system.

The basic pattern of factor segregation and factor interaction in

respeet to the sexual eyele was established for tetrapolar incompatibility by the elassie investigations of Kniep (1920, 1922, 1923, 1928, 1930), Buller (1931, 1941), Brunswik (1924), Vandendries (1936, 1938, *et prior*), Quintanilha (1933, 1939), and others prior to 1940. The system was infused with new interest a little more than a decade ago by the work of Fulton (1950) and Papazian (1950a,b, 1951), who reported several previously unsuspeeted faeets of the system. Of partieular significanee among these were myeelial interaetions, other than dikaryosis, correlated with ineompatibility. Papazian further demonstrated occasional new faetors, found in low frequency among the progeny of crosses and long eonsidered to be mutations (Kniep, 1923), to result from the genetic reeombination of faetor eomponents. Present work has grown more or less direetly from these studies, and the present status of tetrapolar incompatibility ean best be presented as a summary of results from several speeies ineluding *Schizophyllum commune, Coprinus lagopus, Coprinus fimaterius, Pleurotus ostreatus, Collybia velutipes*, etc., among whieh the ineompatibility system differs only in minor detail.

In eaeh tetrapolar speeies, there are two series of ineompatibility factors, *A* and *B*, which segregate independently (Whitehouse, 1949b; Papazian, 1951; Raper and Miles, 1958). An extensive number of alternate faetors in eaeh series exists in the natural population; Whitehouse (1949b) estimates from the earlier literature ea. 100 faetors in each series in most Hymenomycetes and 25–40 in the Gasteromyeetes. In *S. commune*, 121 *A* factors and 61 *B* faetors have now been identified (Raper *et al.*, 1958a,b, 1960; Raper *et al.*, unpublished), and the projected numbers of alternate faetors in the natural population are ca. 340 *A*'s and 64 *B*'s (Raper *et al.*, 1958b). The *A* faetor is known to be constituted of at least two linked subunits, α and β, in *S. commune* (Papazian, 1951; Raper *et al.*, 1958a, 1960), *C. lagopus* (Day, 1960a), *P. ostreatus* (Terakawa, 1957), and *C. velutipes* (Takemaru, 1957a,b, 1961). Linkage between *A* factor subunits may be either very tight or quite loose, e.g., 0.07 crossover units in *C. lagopus* (Day, 1960a) and 0.9–22.8 units in *S. commune* (Raper *et al.*, 1960). In *S. commune*, multiple allelic series of 9 α's and 26 β's have been identified, and, from the repeats in a sample of 33 native *A* faetors, these series are projected to 9 α's and ca. 50 β's for the natural population (Raper *et al.*, 1960, and unpublished). Intrasubunit reeombinants have not been recovered in samples of ca. 125,000 in either *S. commune* (Raper *et al.*, 1960) or *C. lagopus* (P. R. Day, personal eommunieation). The *B* factor of *S. commune* is similarly compound (Raper *et al.*, 1958a), but it eonsists of at least

three subunits (Raper *et al.*, unpublished). Takemaru (1961) reports complex *B* factors in *Lentinus edodes* and *Pleurotus spondoleucus* as well as in *S. commune*. *A* factor specificity is determined by an unique combination of a specific α and a specific β, and the compound factor reacts as a physiological unit; two *A* factors are fully compatible when different for either subunit or for both subunits. *B* factor specificity, activity, and interaction, insofar as they are known, are similarly determined.

When homokaryotic mycelia are grown together, four types of intermycelial reactions are determined by the four possible combinations of *A* and *B* factors.

(1) Unlike *A*'s and unlike *B*'s yield the dikaryon; reciprocal nuclear migration and the formation of clamp connections characterize this, the compatible interaction.

(2) Like *A*, unlike *B*'s interact to form a stable, common-*A* heterokaryon (Papazian, 1950a; Raper and San Antonio, 1954); nuclear migration occurs as in the compatible cross, but clamp connections are not formed (Snider and Raper, 1958; Swiezynski and Day, 1960b).

(3) Unlike *A*'s, like *B* give rise to an unstable, common-*B* heterokaryon (Papazian, 1950b; Fulton, 1950); nuclear migration is severely restricted, and pseudo-clamp connections are formed at the septa (Sweizynski and Day, 1960b; Parag and Raper, 1960; Parag, 1962).

(4) Like *A*, like *B* result, when forced by nonallelic nutritional deficiencies, in the formation of a common *AB* heterokaryon, in which neither nuclear migration occurs nor clamp connections are formed (Swiezynski and Day, 1960b; R. B. Middleton, personal communication).

Of these four types of heterokaryons, the dikaryon regularly produces fruit bodies and basidiospores; infrequent and sporadic fruiting, with normal segregation and recombination, has been reported in each of the other, normally infertile heterokaryons (Brunswik, 1924; Quintanilha, 1939; Takemaru, 1959; Swiezynski and Day, 1960b; Parag and Raper, 1960; Parag, 1962; R. B. Middleton, personal communication).

Fruiting in "illegitimate," i.e., common-factor, combinations often reveals and probably is often the result of mutations of the incompatibility factors or of other loci that disrupt normal function of the incompatibility factors. Thus from common-*A* heterokaryons of *C. lagopus*, Day (1963) obtained several mutations of the *A* factor and an unlinked "suppressor" of *A* factor activity, and from common-*B* crosses of *S. commune*, Parag (1962; Parag and Raper, 1960) recovered mutated *B* factors, although one case was found in which no alteration of the *B* factor occurred, and segregation of mating type was bipolar. The

latter situation was also reported in *Coprinus macrorhizus* by Takemaru (1959). In *S. commune*, mutations at a number of distinct loci, unlinked to either of the incompatibility factors, are the subject of a current study. These "modifiers" profoundly affect many aspects of heterokaryosis, such as nuclear distribution, vegetative vigor, and stability, and prevent fruiting of affected dikaryons. The summed expressions of these mutant alleles almost completely blur the normal distinctions between the different types of heterokaryons. Whether the "modified" phenotypes reflect primary disruption of incompatibility factor functions is still unknown (C. A. Raper, unpublished).

Homokaryotic strains having factor mutations or factor-modifier mutations closely mimic in morphology and behavior the heterokaryotic products of three of the four basic types of mycelial interactions described above. Essentially the same series of mimics results in homokaryotic strains that are disomic for either incompatibility factor or for both factors (Prud'homme and Gans, 1958; Raper and Oettinger, 1962). These relationships are summarized in the accompanying tabulation, where "*" indicates factor mutation and "M," factor-modifier mutation:

Wild type		Mutant mimic[a]	Disomic mimic[a]
Dikaryon	($A^1B^1+A^2B^2$)	A^1—M B^* (Sc)	$A^1A^2B^1B^2$ (Sc)
Common-A heterokaryon	($A^1B^1 : A^1B^2$)	A^1B^* (Sc)	$A^1B^1B^2$ (Cf, Sc)
Common-B heterokaryon	($A^1B^1 : A^2B^1$)	A^*B^1 (Cl)	$A^1A^2B^1$ (Sc)
		A^1—M B^1 (Cl, Sc)	

[a] (Sc) = *Schizophyllum commune*; (Cl) = *Coprinus lagopus*; (Cf) = *Coprinus fimetarius*.

There has been considerable speculation about both the origins and biological significance of tetrapolar incompatibility. It is certainly an effective outbreeding system (Mather, 1942; Whitehouse, 1949b; Papazian, 1951), but the number of alternate factors in each of the two series far exceeds the minimal number that would seriously limit the selective advantage of new factors. Furthermore, the existence of a second series of factors restricting inbreeding to 25% would appear to have little critical advantage in forms having such an efficient means of wide dispersal of spores. The compound structure of the factors is also puzzling and has prompted the suggestion of locus duplication (Day and Holliday, 1959), but for this no direct evidence has been found, although it remains a possibility (Raper *et al.*, 1960). In brief, the probable or even possible evolutionary history of tetrapolar incompatibility is completely obscure, and the selective advantages of the system, though qualitatively obvious, are quantitatively puzzling.

Continuing intensive investigation of the genetics of the incompatibility system per se and of other, associated features of tetrapolar fungi (see Section VI, C, 2) may be expected to yield substantial contributions both in basic genetics and in gene physiology. The fundamental question of the mode of action of the incompatibility factors remains unanswered; a modest beginning in this approach to the problem is suggested in the recent serological examination of minor protein differences correlated with incompatibility factor relationships in S. *commune* (Raper and Esser, 1961).

2. *Heterogenic Incompatibility*

Heterogenic incompatibility is far less well known than homogenic incompatibility and has been worked out in detail in only a single fungus, although there is evidence that such systems may be of widespread occurrence (Esser, 1962). In *Podospora anserina*, a secondarily homothallic Ascomycete with 2-allele homogenic incompatibility, Esser (1954a, b, 1956, 1959a, b) has discovered and analyzed an additional genetic system that serves to restrict or to prevent cross-mating between individuals of different geographical races. This restrictive system, designated heterogenic incompatibility by its author, consists of alternate alleles at each of four loci and regulates mating competence as follows: compatible (+) and (−) strains homozygous for all four loci interact reciprocally, but strains heterozygous for these loci in different combinations either interact unilaterally or fail to interact. The operation of heterogenic incompatibility in *P. anserina* is illustrated in Fig. 11. Further genetic and biochemical investigations have shown that the action of the genes responsible for heterogenic incompatibility is not limited to the sexual process. It is manifest also in vegetative heterokaryons. By means of specific effects emanating from certain genes, the reproductive capacity of nuclei carrying certain other genes of the system is suppressed. These interactions find their physiological expression in an alteration of protein specificity in the heterokaryon as demonstrated by immunological methods.

Comparable restrictions to cross-mating between geographical races occur in the Ascomycetes *Sordaria fimicola* (Olive, 1956; Carr and Olive, 1959) and S. *macrospora* (Esser, unpublished) and in the Basidiomycetes *Coprinus micaceus* (Vandendries, 1927, 1929) and *Polyporus betulinus* (J. H. Burnett, personal communication).

Such restrictions to interracial breeding serve as an isolating device and may be an important factor in speciation. Heterogenic incompatibility thus plays a role exactly opposed to that of homogenic incompati-

bility. Heterogenic incompatibility limits the sexual compatibility of heterogenic raees and promotes inbreeding. By the restriction of sexual interaction to members of the same race, the race thus becomes the evolutionary unit. Homogenic incompatibility, on the other hand, restricts the sexual proeess between genetieally similar organisms and guarantees that the speeies *in toto* participates in the evolutionary proeess. Far more information relating to the occurrence, distribution, and genetics of

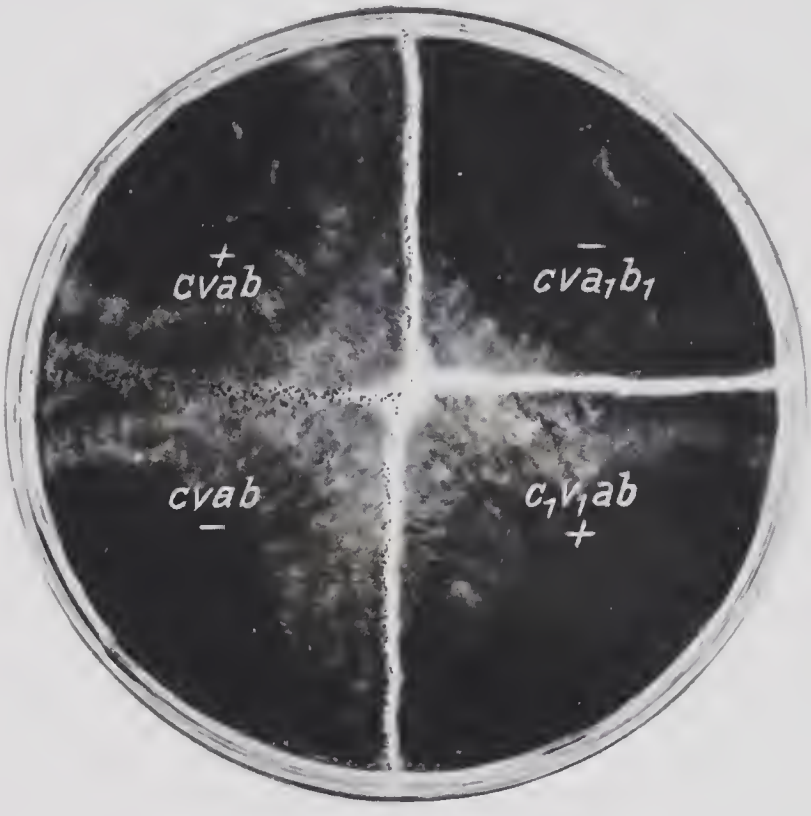

Fig. 11. The operation of heterogenic incompatibility in *Podospora anserina*. From Esser (1956).

interrace sterility and heterogenic incompatibility is required properly to assess their overall signifieance in the fungi.

B. Heterokaryosis

Heterokaryosis, the association of genetically different nuclei in a system capable of propagation, oceurs commonly in and is unique to the fungi. The association of diverse, i.e., (+) and (−), nuclei was suspected by Blakeslee (1906) as the underlying cause of "neutral mycelia," unstable, nonreactive mycelia subject to vegetative sectoring into (+) and (−) sectors that originated occasionally from germsporangial spores of *Phycomyces nitrens* of the Mueorales. This was subsequently proved by Burgeff (1914), who, by grafting together

sporangiophores of (+) and (−) strains, recreated "neutral mycelia" in the laboratory. Burgeff coined the terms "heterokaryon" and "heterokaryosis" and worked extensively with numerous heterokaryotic combinations between different strains of several species of the Mucorales. This early history of heterokaryosis has often been ignored in recent years (Bistis, 1960). Heterokaryosis was "rediscovered" by Hanson and Smith in 1932 as the basis for variability and vegetative sectoring in an imperfect fungus, *Botrytis cinerea*. Since this time, heterokaryosis has gained wide recognition both as a significant biological phenomenon of very widespread occurrence among fungi and as an experimental tool in fungal genetics. An extensive literature is concerned with the occurrence, characterization, biological significance, and utility of heterokaryons, and numerous reviews and critical treatments are available (Beadle and Coonradt, 1944; Pontecorvo, 1946; 1953; Jinks, 1952; Whitehouse, 1951; Sansome, 1946a; Raper and San Antonio, 1954; Raper, 1955).

The biological significance of heterokaryosis follows from its roles in three different phenomena (Raper, 1955): (1) it provides a means of rapid adaptation to specific environmental situations; (2) it permits, via complementation, the restoration of normal function by association of deficient genomes; and (3) it has become an integral component (the dikaryon) in the life cycle of the higher fungi.

Heterokaryosis is known to occur among the Phycomycetes only in the Mucorales, and its occurrence in the Protoascomycetes is doubtful. In the Euascomycetes and in the Fungi Imperfecti, it is known to be very common, and it may be universally present in these groups, as it most certainly is in all groups of the Basidiomycetes.

Heterokaryons vary widely in respect to many features: (1) Origin may be either by hyphal fusion (Buller, 1931; Dowding and Buller, 1940; Dodge *et al.*, 1945), by incorporation of dissimilar nuclei in ascospores of secondarily homothallic species of Ascomycetes (Dodge, 1927, 1928a; Rizet and Engelmann, 1949) and in basidiospores of secondarily homothallic Basidiomycetes (Sass, 1929; Skolko, 1944), by abnormal cleavage of ascospores of normal heterothallic species (Lindegren, 1934; Sansome, 1946b), or by multinucleate cleavage of germ sporangiospores (Blakeslee, 1906; Burgeff, 1912, 1914). (2) Extent varies from severely restricted to the immediate line of mycelial intermingling (Papazian, 1950b; Raper and Fennell, 1953) to the complete heterokaryotic conversion, via nuclear migration, of the original homokaryotic mycelia (Buller, 1931; Dowding and Buller, 1940; Rizet and Engelmann, 1949). (3) Stability ranges from the perennial dikaryotic mycelium of certain Basidiomycetes, from which components may be recovered—except for

uninucleate asexual spores in some species (Brodie, 1936; Nobles, 1935) —only by microsurgery (Harder, 1926; Fries and Aschan, 1952; Papazian, 1955) or by treatment with chemical agents (Miles and Raper, 1956), to associations that sector with great frequency and regularity (Pontecorvo, 1946). (4) Continuity of the association is always lost in sexual reproduction; it may be retained in asexual reproduction only in species that produce multinucleate spores (Hansen and Smith, 1932; Gossop *et al.*, 1940). (5) The nuclear ratio is either unity, in dikaryons, in which the ratio is maintained by conjugate division, or indefinite, in all other heterokaryons, in which the ratio ranges widely and appears to be determined in many cases largely by the input ratio of component nuclei (Prout *et al.*, 1953; Pittenger *et al.*, 1955; Pittenger and Atwood, 1956) rather than by selection of hyphal elements that contain a favorable ratio of nuclear types (Beadle and Coonradt, 1944).

The genetic control of heterokaryotic characteristics appears to be of at least two types: incompatibility factors *per se* and other genetic determinants that have recently been termed incompatibility factors, in disregard of the common use of the term to apply to factors restricting sexual mating. (1) The basic role of incompatibility factors *sensu strictu* in heterokaryosis can be exemplified by three examples: In *Neurospora crassa,* facile heterokaryosis occurs only between strains of the same incompatibility class, i.e., *A* and *A* or *a* and *a,* whereas heterokaryons are established only with difficulty between compatible strains, i.e., *A* and *a* (Beadle and Coonradt, 1944; Sansome, 1946a; Gross, 1950). In the related secondarily homothallic species, *Neurospora tetrasperma* and *Podospora anserina,* however, heterokaryosis readily occurs between compatible strains, i.e., *A* and *a* (Dodge *et al.*, 1945; Rizet and Engelmann, 1949), but in the latter species, heterokaryosis may be prevented by the genes responsible for heterogenic incompatibility (Esser, 1959a). In the tetrapolar Basidiomycetes, the four incompatibility relationships predetermine four exactly specific types of heterokaryons (see above). As pointed out previously (Raper, 1955), there is a very nice correlation throughout the fungi between incompatibility systems and "allowable" heterokaryons. (2) There have been reported in the past few years for *Neurospora crassa* a number of additional genetic factors, nuclear and cytoplasmic and *characteristic only of specific strains,* which profoundly affect the establishment and characteristics of heterokaryons between strains of like mating type (Holloway, 1955; Garnjobst, 1953, 1955; Garnjobst and Wilson, 1956; Wilson *et al.*, 1961; Pittenger and Brawner, 1961).

[Parenthetically, the present authors regret the increasing use of the

terms "compatibility" and "incompatibility" in a strictly heterokaryotic connotation. Viewed with historical perspective, heterokaryons in *Neurospora crassa* best succeed when constituted of incompatible, compatible strains (translation: sexually incompatible, heterokaryotically compatible). Unless one chooses to ignore the entire baekground of the work on *Neurospora*, the indiseriminate use of these terms—without even the modifying adjeetives "sexual" and "heterokaryotie"—renders much of the text of certain recent papers in this field a close approach to scientific double-talk.]

Finally, heterokaryosis provides, through the protraeted assoeiation of genetieally diverse genomes, the essential first step in the eryptic sexual processes of parasexuality and somatie recombination.

C. Parasexuality and Somatic Recombination

One of the most significant recent developments in fungal genetics is the discovery and detailed analysis by Pontecorvo, Roper, and their associates (for early references, see Ponteeorvo, 1953) of diploidy and recombinational processes in the vegetative mycelium of *Aspergillus nidulans* and the subsequent revelation of comparable proeesses in a number of Ascomyeetes, Fungi Imperfecti, and Basidiomycetes. Somatic recombination, earlier described in somatic tissue of the fruit fly *Drosophila* by Stern (1936), has particular significance in the fungi, since the recombinant genomes beeome a part of the genetic continuum in these organisms.

It now appears likely that different basic modes of somatic recombination apply to the Ascomycetes and the associated Fungi Imperfecti on the one hand and to the Basidiomycetes on the other. In the former, the *parasexual cycle* (Pontecorvo, 1956) appears to be characteristic. In the latter, mitotic reeombination has been reported in only one group, the smuts; otherwise, in two other groups, the rusts and the Hymenomycetes, the mechanism(s) of recombination is not known, and no evidence has yet been found in these forms of certain essential features of parasexuality.

1. The Parasexual Cycle

The parasexual cycle is well known and requires only the briefest summary here for comparison with recombinational processes reported in the Basidiomycetes. The main events in the parasexual cycle (for details: Pontecorvo, 1958) are: (1) heterokaryosis, (2) rare fusion (ca. 10^{-6}) of heterokaryotic nuclei to form heterozygous diploid nuclei, (3) oecasional (ca. 10^{-2} per nucleus per generation) mitotic crossing

over to yield partial homozygous diploid nuclei, and (4) constant haploidization (with a frequency of ca. 10^{-3} per nuclear division) via successive stages of aneuploidy. A recent paper of Käfer (1961) shows nondisjunctional irregularities to be somewhat more frequent than previously known and to lead to a wide variety of aneuploid states. The haploid products of the parasexual cycle may be recombinant only for whole linkage groups, or they may be additionally recombinant for a part of one linkage group, depending upon the previous occurrence of mitotic crossing over prior to haploidization. Since the rarity of mitotic crossing over effectively precludes multiple exchanges per diploid nucleus, linkage relationships are quite characteristic and different from those revealed in meiotic recombination.

2. Somatic Recombination in Basidiomycetes

Somatic recombination has been reported to date in three different groups of the Basidiomycetes, the smut fungi, the rust fungi, and the Hymenomycetes. The study of somatic recombination in these forms has its special methods as well as its peculiar problems because of the more complex life cycles and especially because of the fact of parasitism in the smuts and rusts.

In the smut, *Ustilago maydis*, Holliday (1961b) has reported the isolation and analysis of two, multiply marked diploids. From the types of segregants obtained from these, he concluded that their genotypes were "consistent with the view that they arose as a result of mitotic crossing-over."

Otherwise, in the rusts and Hymenomycetes, the work to date has been primarily concerned with recombination in the vegetative dikaryon, and, with one exception to be noted below, has used living host plants or tester strains of the same species as selecting agents for recombinants in the rusts and Hymenomycetes, respectively. That the selective agent may not be strictly neutral has added an interesting facet to these studies.

Among the rust fungi, somatic recombination has been reported in two different species of *Puccinia*. From infections of mixed dikaryons of *P. recondita* f. *tritici*, numerous new dikaryotic races, each exhibiting certain characteristics of both dikaryotic parents, have been isolated and characterized by Vakili and Caldwell (1957). Comparable and more extensive results have been reported for *P. graminis* f. *tritici* by Bridgmon (1959), Watson (1957, 1958), who termed the phenomenon "somatic hybridization," and Ellingboe (1961). Ellingboe isolated numerous novel, dikaryotic races, selected for uredial color, from several

different crosses and found the recombinants in each case to vary widely in pathogenicity to test strains of the host; 15 isolates from one cross were all distinct. The indicated number of recombinational types appears to be far larger than the largest samples analyzed to date and the results can hardly be explained except in terms of genetic recombination between entire genomes. Ellingboe (1961) has suggested that the recombinational mechanism is meiosis-like in nature.

Somatic recombination has been examined somewhat more intensively in *Schizophyllum* and *Coprinus* of the Hymenomycetes, in which the Buller phenomenon (Buller, 1931; Quintanilha, 1939), the dikaryotization of a homokaryon by an established dikaryon, has most often served as the means of selection of recombinant genotypes. In dikaryotic-homokaryotic, or di-mon, matings of tetrapolar species, dikaryotization of the homokaryon occurs rapidly and regularly when the homokaryon is compatible with one or both of the components of the dikaryon (legitimate), e.g., $A^1B^1 \times (A^2B^2 + A^3B^3)$ and $A^1B^1 \times (A^1B^1 + A^2B^2)$, and more slowly and sporadically when the homokaryon is compatible with neither of the dikaryotic components (illegitimate), e.g., $A^1B^2 \times (A^1B^1 + A^2B^2)$. Two alternate phenomena have been demonstrated to be responsible for dikaryotization in illegitimate di-mon matings: (a) the migration of nuclei of both dikaryotic components and the reestablishment of the original dikaryon at the growing periphery of the homokaryon (Papazian, 1950b, 1954; Crowe, 1960) and (b) the exchange of nuclear components and the reconstitution of a nuclear type that is compatible with that of the homokaryon, i.e., somatic recombination. The latter phenomenon was first observed by Quintanilha (1939), who considered it a simple exchange of nuclear components, probably chromosomes, during conjugate division or in nuclear migration. The presence of nuclei recombinant for mating type in derived dikaryons of illegitimate di-mon matings has been repeatedly confirmed (Papazian, 1951, 1954; Gans and Prud'homme, 1958; Kimura, 1958; Swiezynski, 1962; Ellingboe and Raper, 1962). From studies with legitimate and illegitimate di-mon systems, the process of recombination is now known to involve: (1) independent assortment of incompatibility factors (Papazian, 1954; Gans and Prud'homme, 1958; Crowe, 1960); (2) crossover recombination of incompatibility factors and linked markers (Crowe, 1958, 1960); (3) crossover recombination of α and β subunits of the *A* factor (Parag, 1962; Raper, 1961); and (4) recombination by independent assortment and by crossing over of nonselective markers (Ellingboe and Raper, 1962).

Analysis of recombinants via progeny of derived dikaryons is

laborious, and most of these studies have involved samples of very modest size. Two of the studies, both with S. *commune*, have used other means of analysis. (1) Parag (1962; Raper, 1961) has examined dikaryons for recombination, in the absence of a homokaryotic selecting strain, by total sample isolation of macerated fragments followed by the identifieation of incompatibility factors in appropriate di-mon matings (Papazian, 1950b). Recombinants for mating type occurred in thc "unmated" dikaryon in a frequency of the order of 1%. (2) Ellingboe and Raper (1962) have used heterokaryotic allelic tests for nonselective bioehemical markers to analyze recombinant components of dikaryons derived from illegitimate di-mon matings. This latter study has revealed a wholly unsuspected situation that clearly demonstrates the involvement of the selecting homokaryotic strain in the recombinational process. In a sample of 178 reeombinant strains, selected for recombinant mating type and derived from isogenic (6–10 backcrossed generations), multiply marked, illegitimate di-mon matings, only 79 carried nonselective markers from either or both of the dikaryotic components. In these recombinants, linkage data strongly resembled those in meiotic recombination rather than those expected from mitotic recombination. The remaining 99 strains earried only the recombined incompatibility factors from the members of the dikaryon; otherwise, thcir nonselective markers were derived exclusively from the homokaryotic selector strain. Two types of recombinational events are thus indicated: (1) a meiosis-like event in the dikaryon, and (2) a subsequent event of unknown nature in the homokaryon. The latter could be (*a*) a second stage of two successive events in the two mycelial components, (*b*) a triple fusion of the two dikaryotic nuclei and the homokaryotic nucleus, or (*c*) a recombinational event of specific and unknown nature that recombines incompatibility factors without involving the whole genomes of the participating nuclei —this latter possibility was favored on the basis of the available evidence. Data recently reported by Ellingboe (1963) clearly indicate a mechanism of exchange specific for the incompatibility factors: in each of 20 cases, A_α and A_β subunits were transferred together to the basic genome of the selector without transfer of the internal marker *pab*. Other, and less compclling, evidence that thc selector strain and the migration of nuclci are implicatcd in somatic recombination has been reportcd in *Coprinus* by Kimura (1958) and Swiezynski (1962).

In a search for genetic recombination betwcen genomes of two distinct dikaryons when grown together, only exchange of whole nuclei could be found in *Coprinus lagopus* (Swiezynski, 1962).

Thc nced for additional information to clarify the present uncertain

status of somatic recombination in the Hymenomycetes is all too evident. Particularly needed are new methods for the selection, recognition, and characterization of recombinant genotypes in sufficient number to provide definite genetic evidence of the underlying mechanism(s) of recombination.

VII. Concluding Remarks

In the foregoing treatment of the fungi, the authors have tried to achieve two objectives: (1) to weave into a generalized description of the fungi detailed accounts of those features of morphology, physiology, and development that are peculiarly characteristic of the fungi and that account for the extensive use of fungal materials in the investigation of a wide array of basic biological phenomena; and (2) to summarize the contributions of studies with fungi to the recent, rapid expansion of a single area of universal significance, genetics. Time and space, regrettably, did not permit the inclusion of a third facet, which, though still fragmentary, holds exciting promise for the future: the underlying basis of differentiative development. The morphogenetic chain from gene through biochemical and physiological adjustment to cellular, tissue, and organ differentiation remains one of biology's least understood basic phenomena. The initial stages in this sequence, from gene to specific biochemical product, is presently the subject of major experimental endeavor with both bacteria and fungi, and in a number of specific cases, such as the *td* locus in *Neurospora* and in *E. coli*, the consequences of genetic change on the characteristics of the enzyme product are reasonably well known, though not in the desired detail. Later stages in the sequence, from biochemical cause to morphological effect, have been less intensively studied in microorganisms, and the available information is correspondingly less extensive and more fragmentary. Such information, however, is not inconsiderable, and intimations of the possibilities inherent in fungal studies can be seen in numerous studies reported here in other connections, e.g., sexual hormones, mutative disruption of sexual expression, etc., but in which knowledge of essential stages in an orderly progression is wanting. Consideration of more definitive studies, which, however, lack compelling direct relevance to our primary objectives, has not been attempted. In this latter category belong such investigations as that relating the biochemical details of metabolism to vegetative or reproductive differentiation in the aquatic fungus *Blastocladiella* (Cantino, 1961). Coverage of this additional aspect of experimental mycology would have given heightened plausibility to the expectation that, of all groups of organisms, the fungi

provide the most favorable material for the eventual elucidation of the complete step-by-step progression of events from gene to final differentiated form.

Otherwise, the account given here reflects the special interests and areas of competence of its authors. Little emphasis has been given the fact that fungi are of extreme importance in the balance of nature and to the welfare of man. The degradative competence and the synthetic abilities of fungi were only briefly mentioned in passing. Obviously, the choice of topics for intensive treatment was dictated neither by considerations of the practical benefits in foods, drugs, spirits, and other desirable products of native and domesticated fungi nor by an esoteric appreciation of the fact that the organic clutter about us is only of recent origin. The "blue and green molds" of the genus *Penicillium*, for example, are versatile in the extreme: they delight the palate of the gourmet in Roquefort, Camembert, Gorgonzola, and Stilton cheeses; they annoy the fastidious by their omnivorous decay of fruits, vegetables, and animal remains; they cure the ill with penicillin, a by-product of their weird metabolism; they perplex the biochemist and enchant the manufacturer by the array and quantity of unusual and marketable compounds that they can synthesize from simple and cheap material; and they confound the taxonomist by their variability and intergradations. Yet the *Penicillium* molds are not particularly exceptional: they happen to be quite common, and the results of their activities are fairly evident. Many groups of fungi are equally versatile. There are, however, well-known groups of specialists: plant and animal parasites—even to forms that grow only upon the leading edge of the left wing case of a single species of beetle.

The study of fungi is in process of transition and dichotomy. Early mycology was almost wholly taxonomic, and taxonomy remains an important aspect for study. Experimental mycology, however, is in rapid growth and encompasses both the study of fungi per se and the use of fungi as material for the elucidation of many processes and features common to all living things. Too often, perhaps, the overall biology of the fungal material is overlooked in the detailed examination of a single facet of its activity. But, in the laboratory as in nature, the fungi serve us well.

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CHAPTER 4

The Plant Cell: Aspects of Its Form and Function

By BRUCE R. VOELLER

with Electron Micrographs

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I. Introduction

In contrast with the immense diversity in the appearances and in the special environmental adaptation of the various groups of plants and animals, the similarity in the form and mode of function of the components of their living cells is startling and impressive. With few exceptions (e.g., the blue-green algae), living cells contain such structures as mitochondria, Golgi bodies (dictyosomes), ribosomes (ribonucleoprotein particles), the so-called "endoplasmic reticulum," and a nucleus with its various familiar components. The similarity of the fine structure of these cellular components, as revealed in the electron microscope, bespeaks their common nature in very different groups of

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organisms. Moreover, an impressive body of biochemical information confirms this common nature.

The preceding volumes of this series have contained detailed studies of the form and function of many of the cellular components common to both plants and animals. Therefore, in the present chapter, these cellular constituents will be discussed only briefly, with special reference to plants. Most of the chapter will be devoted to consideration of a few selected plant cell structures which either for important physiological reasons are distinctly different from those generally observed in animal cells, or which have long been a source of controversy that can now be fairly well resolved.

Two of the most strikingly characteristic plant cell structures, the cell wall and the chloroplast, have already been considered in detail in this series, respectively by Mühlethaler (1961) and Granick (1961). The present chapter will therefore deal chiefly with the plasmodesmata, the dictyosomes (Golgi apparatus), and the vacuoles of plants.

The chapter will include information from nearly all groups of plants but should not, in the main, be regarded as encompassing the blue-green algae, bacteria, and fungi. Thus, generalizations made below for "*plant cells*" should not necessarily be considered appropriate to these latter three groups of plants. Of the three special groups, the bacteria and fungi have been discussed in other chapters (Robinow, 1960; Raper and Esser, 1963). For comparison with the more familiar cells of other groups of plants, the blue-green algal cell will be briefly discussed in the present chapter.

At the time of writing this review, the spring of 1962, many of the classical cytological problems are beginning to be resolved through use of the electron microscope. Initial difficulties in fixing plant cells with osmic acid and in sectioning tissues with cell walls have now been overcome, although the new techniques are not yet in general use. Moreover, interest in cell physiology, against the background of the whole plant in its environment, has been revived by the recent ascendancy of microchemical methods and of biochemical studies. Thus, the task of accurately presenting a picture of the plant cell, its form and function, is made both stimulating and difficult by the rapid increase of information in print. Notwithstanding, it is hoped that the present chapter, in conjunction with those of Granick, of Mühlethaler, and of Raper and Esser, will be of use in bringing together some of the immense literature relating to the structure and physiology of plant cells and will give a general idea of current conceptualization of the living plant cell.

II. The Plant Cell

A. *The Generalized Plant Cell*

Figures 1–3 illustrate sections through representative plant cells. In them can be seen most of the cellular components characteristically observed, including mitochondria, proplastids, dictyosomes, nuclei, ribosomes, endoplasmic reticulum, and cell membranes.

The mitochondria characteristically are ovoid to round organelles (Figs. 1–3), although they also can be very irregular in shape, as often seen in animal cells. In all groups of plants the mitochondria are bounded by a double membrane, the inner unit of which is continuous with the platelike cristae (in land plants) or filamentous villi (in many algae), which penetrate into the interior of the mitochondrion, as shown in Fig. 4. The biochemistry and physiology of plant mitochondria are fundamentally quite similar to those of animal cells, although differences do exist (cf. Crane, 1961; Goddard and Stafford, 1954; Hackett, 1959; Millerd, 1956; Novikoff, 1961a).

The nuclei of plant cells are quite variable in size and shape, but most typically spherical. At least in some algae, the nuclei have been reported to rotate at remarkable rates. Valkanov (1933) reported that the nuclei of *Nitella* and *Tolypellopsis* made a full revolution in 10–20 seconds. Fetzmann (1958) confirmed Valkanov's observation and stated that in cells of *Chara foetida* nuclei rotated as rapidly as once every 1.5–2 seconds, about the speed of a long-playing phonograph record.

The nucleus is surrounded by a double membrane with numerous pores (Figs. 1–3, 13). In appropriately prepared animal cells, these pores, and the nuclear and cytoplasmic regions immediately adjacent to them, appear to contain electron-dense material (Merriam, 1961; cf. also Mirsky and Osawa, 1961). Similar material has also been observed in the nuclear pores of osmium-fixed plant cells, including desmids (Drawert and Mix, 1961) and probably cells from flowering plants (e.g., see Fig. 2, Heitz, 1957). This material is not, however, generally visible following permanganate fixation (Drawert and Mix, 1961), although occasionally the pore region appears lighter than adjacent regions (e.g., see Fig. 2 in Mollenhauer *et al.*, 1960). The nucleolus (Fig. 1) is not bounded by a membrane and, like the nucleoplasm, appears to be composed of small granules; these are morphologically indistinguishable, other than by their localization, from cytoplasmic ribosomes (Figs. 1, 4, and 10). Moreover, these particles apparently have physical and chemical properties which are similar in many respects to those of ribosomes in the cytoplasm (Bonner, 1961: Frenster *et al.*, 1960;

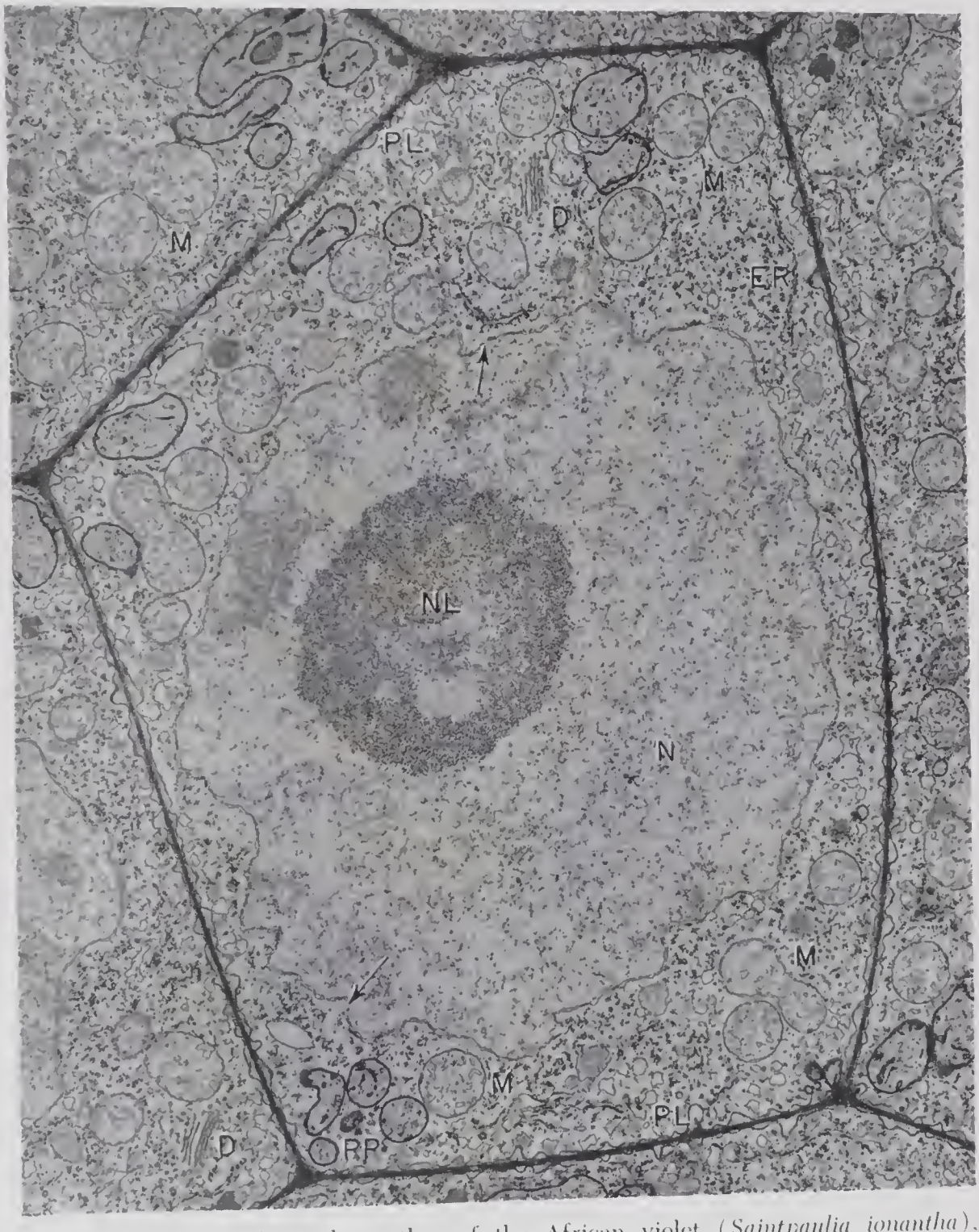


FIG. 1. A cell from the anther of the African violet (*Saintpaulia ionantha*), showing representative contents of a plant cell. Fixation in osmic acid. Mitochondria (*M*), dictyosomes (*D*), endoplasmic reticulum (*ER*), a nucleus (*N*), a nucleolus (*NL*), the plasmalemma (*PL*), and proplastids (*PP*) are illustrated. Arrows indicate "nuclear pores." Magnification: × 6264. (Electron micrograph by Ledbetter and Porter.)

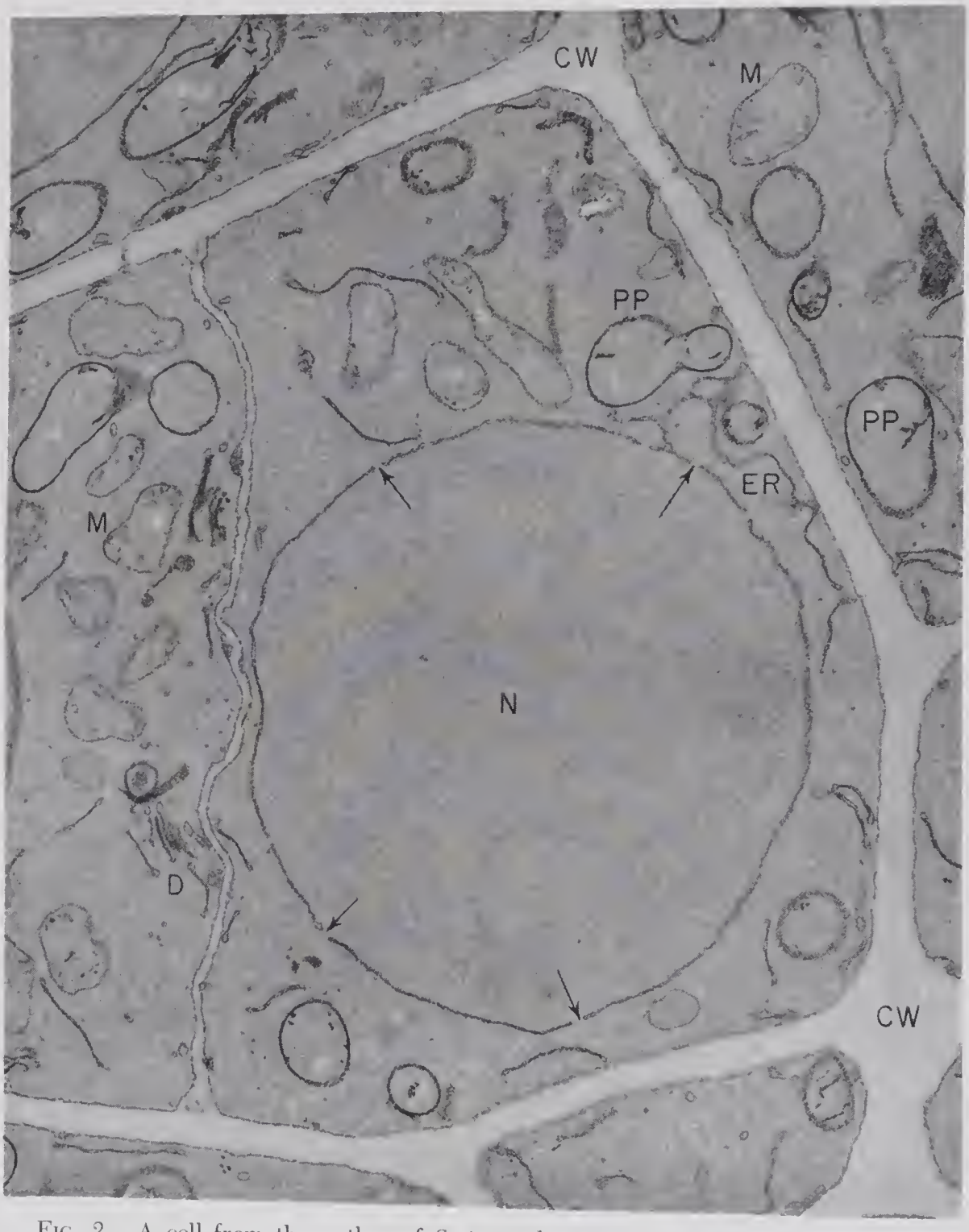


FIG. 2. A cell from the anther of *S. ionantha*. Fixation in potassium permanganate. "Nuclear pores" may be readily seen (arrows). Also visible are the nucleus (*N*), proplastids (*PP*), mitochondria (*M*), dictyosomes (*D*), and the cell wall (*CW*). Note the graphic simplicity in the appearance of the contents of this permanganate-fixed cell (also Fig. 3) in comparison with the wealth of detail visible in Fig. 1. Magnification: × 8700. (Electron micrograph by Ledbetter and Porter.)

FIG. 3. Permanganate-fixed cell from the anther of *S. ionantha*. "Nuclear pores" (arrows) and proliferation of the nuclear membrane in relation to the endoplasmic reticulum (*ER*) are shown. Also visible are a dictyosome (*D*), in planar view, mitochondria (*M*), two nuclei (*N*), proplastids (*PP*), and plasmodesmata (*Pd*). Magnification: × 11,418. (Electron micrograph by Ledbetter and Porter.)

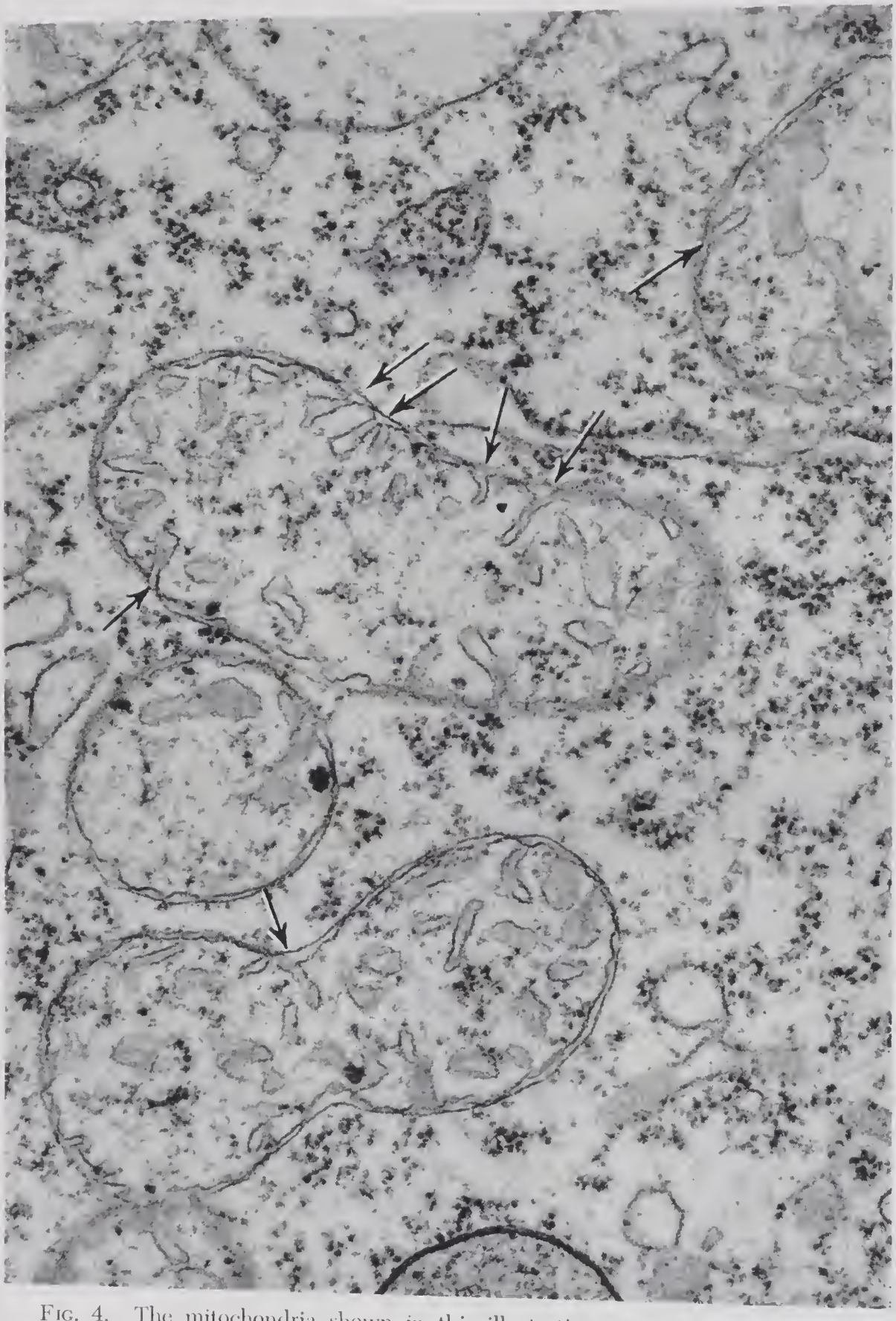


FIG. 4. The mitochondria shown in this illustration are surrounded by double membranes. The "cristae" are seen to be projections of the inner portion of the double membrane. Osmium-fixed cell from an anther of *S. ionantha*. Magnification: × 52,000. (Electron micrograph by Ledbetter and Porter.)

Ts'o and Sato, 1959). Particularly in permanganate-fixed tissues, the outer membrane of the nucleus is often continuous with elements of the endoplasmic reticulum (Figs. 2, 3, and 13). In some cases elaborate proliferations from the nuclear membrane have been observed (Whaley *et al.*, 1960, 1961), which quite probably are artifactual (Mollenhauer *et al.*, 1960). However, these proliferations are much less common after fixation in solutions of osmium tetroxide. In a recent study, Mühlethaler and Bell (1962) reported that the nuclear membranes of plastid-free egg cells of the bracken fern (*Pteridium*) undergo two elaborate proliferation phases. The vesicles formed in the first phase are reported to differentiate into mitochondria; those of the second phase become proplastids. It is clear that such a series of events comprises a radical departure from generally known cytological phenomena. Thus, Mühlethaler and Bell's interesting work should be accepted with considerable caution until a thorough and detailed study of bracken oospores has been completed and confirmed. For detailed consideration of the nucleus in general, the reader is referred to Bahr (1956), Serra (1955a, b, c), and Mirsky and Osawa (1961).

At this point it may profitably be noted that tissues fixed in potassium permanganate solutions show rather different fine structure from those fixed in osmium tetroxide solutions. The lamellar structures such as the nuclear, mitochondrial, and plasma membranes, the dictyosomes, and the endoplasmic reticulum are very flat, smooth and even, after permanganate fixation (Figs. 2 and 3) and give a highly diagrammatic impression. Moreover, the ribosomal particles and very frequently the nucleoli are not visible after permanganate treatment. Also, the endoplasmic reticulum seems much more condensed. Osmium-fixed cells, by contrast, are very rich in detail (Figs. 1, 4, and 10), and quite well-defined nucleoli and abundant ribosomes are visible. Wrischer (1960) studied the effects of X-rays, ultraviolet light, CO_2, N_2, KCN, etc., upon plant dictyosomes and endoplasmic reticula. He showed that control tissues fixed in osmic acid contained flat, straight lamellae (as observed by various workers), whereas experimentally damaged tissues contained strikingly curled or cup-shaped (sometimes actually circular) dictyosome profiles. Thus, the possibility should be considered that the similar curved profiles generally seen in permanganate-fixed cells may also be a consequence of injury during fixation. However, it is at present difficult to say with complete certainty which of the two techniques provides the more accurate representation of living cells. But, the extensive studies of M. C. Ledbetter and K. R. Porter, of Harvard University, lead them definitely to favor osmium (personal communication).

Lysosome-like structures, which are not visible in the present illustrations, have been reported in a number of plants (Genèves *et al.*, 1958; Lund *et al.*, 1958). They appear as round to oval structures, 0.1–0.3 × 0.2–0.6 μ (Genèves *et al.* 1958). Thus, they are roughly the same size as (a little smaller than) mitochondria. However, they are more electron dense than mitochondria and most other structures. They also differ in having a single, limiting membrane. In addition, they appear in the mitochondrial fraction of ultracentrifugates (Lund *et al.*, 1958), as is the case with animal cells. But, they have not generally been observed in plants by other workers. Nor have the appropriate biochemical and enzymological studies been carried out to demonstrate that the lysosome-like structures are functionally related to those of animal cells (cf. the discussion of animal cell lysosomes by Novikoff, 1961b).

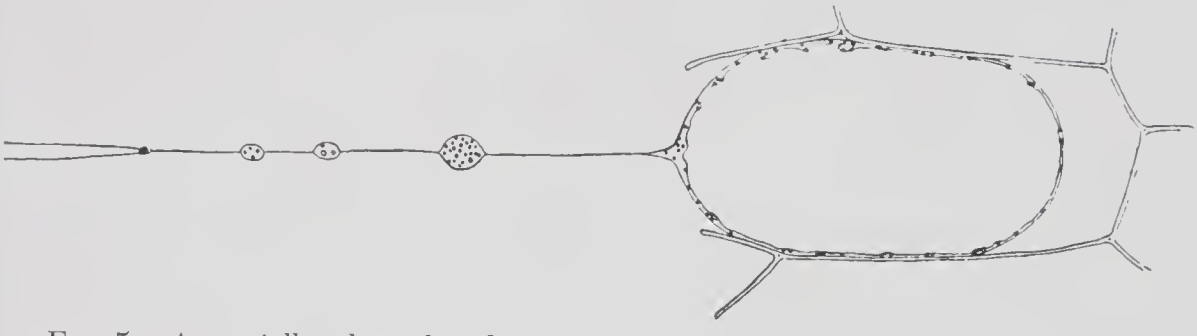


FIG. 5. A partially plasmolyzed onion epidermal cell, with a very large central vacuole. The end of the cell wall has been removed. The plasmalemma has been drawn out by means of a microneedle. Small globules of cytoplasm have been trapped between the two stretched layers of the plasmalemma. From Plowe (1931a).

The ribosomes of plants are small particles (ca. 125–150 A.) which are scattered in groups or clusters throughout the cytoplasm (Figs. 1, 4, and 10). They often are seen loosely covering the outside of elements of the endoplasmic reticulum and the outer side of the outer membrane of the nucleus, as in animal cells. Moreover, the physical and chemical properties which have been investigated seem to be similar in many respects to those of animal cells (Bonner, 1961; Lund *et al.*, 1958; Szarkowski *et al.*, 1960; Ts'o, 1958; Ts'o *et al.*, 1956; Webster, 1955; Webster and Lingrel, 1961). Thus, it is reasonable to suppose that plant ribosomes play a role in protein synthesis analogous to that much more fully studied in animal cells and in bacteria.

The existence of the plasmalemma, or cell membrane, which is clearly recognizable in Fig. 5, has been known for a very long time. It was seen and recognized as such during the middle of the nineteenth century, especially in plasmolytic studies in which the membrane retracts from the cell wall. For the last sixty years the plasmalemma has

been the subject of innumerable electrical, permeability, and surface tension studies. It was particularly fully examined by Chambers and his colleagues (cf. Chambers and Chambers, 1961). They carried out many microdissection studies and investigated, by means of microinjection, the movement of dyes and hydrogen ion indicators across the plasmalemma. They showed that cells cultured in media containing various dyes did not become stained. When the same dyes were microinjected into the cytoplasm of cells, however, the dyes diffused throughout the cytoplasm. Thus, it was concluded that the plasmalemma, not the cytoplasm as a whole, acted as a barrier to movement of the dyes.

The cell membrane, as shown in Figs. 1, 10, and 16, appears as a single membrane in the electron microscope. Thus, it resembles the single membrane surrounding vacuoles (Fig. 16) but differs from the double membranes of mitochondria, plastids of various sorts, and the nucleus. Like the vacuolar membrane, it appears to be ca. 70–100 A. thick, in agreement with the estimate (100 A.) of Remington (1928) based upon electrical capacitance and resistance. Under some conditions in osmium-fixed material, the plasmalemma has an irregular, somewhat infolded appearance, which might be related to a process similar to pinocytosis (Buvat, 1961; Buvat and Lance, 1957), although there is not yet satisfactory evidence in support of this view.

The endoplasmic reticulum, which can be seen in most of the present illustrations, is found in all groups of plants presently under discussion. As already noted, it is frequently connected with the nuclear membrane. It also is very commonly observed in close proximity to plasmodesmata (see below). However, there is no published evidence

FIG. 6. *Phormidium* sp. An electron micrograph of an entire filament. Permanganate fixation. Magnification: × 8500. From W. T. Hall (unpublished).

of its role in plants, in contrast with that proposed for animals (e.g., see Porter, 1961).

B. The Blue-Green Algae

In contrast with most other plants, including other algae, the blue-green algae show remarkably little intracellular differentiation (Figs. 6 and 7). Study with the electron microscope has largely confirmed earlier light microscope observations of the absence from blue-green algae of recognizable plastids, mitochondria, true vacuoles, or membrane-bounded nuclei with chromosomes. Indeed, classical studies mainly distinguished central and cortical regions in these cells. However, electron micrographs

FIG. 7. *Phormidium* sp., illustrating the lamellar membrane system present in the chromatoplasm. Permanganate fixation. Magnification: × 31,000. From W. T. Hall (unpublished).

do reveal the presence of additional structural organization, including the presence of an extensive lamellar membrane system, of 100–150 A. diameter ribosomes and of a 70 A.-thick cell membrane (actually known to exist from earlier studies) similar to that of higher organisms (see, e.g., Ris and Singh, 1961). The lamellar membrane system has also been demonstrated in recent light microscope studies (Geitler, 1958, 1960).

The central zone, or centroplasm, of the cell has been regarded as chromatin-like since the turn of the century (cf. Fritsch, 1959) and has been shown in many species to stain in the Feulgen test (Petter, 1933; Spearing, 1937; Ris and Singh, 1961). The distribution and appearance of the centroplasm are quite variable and the relative proportions of centroplasm and cortical cytoplasm, or chromatoplasm, can markedly vary, even from cell to cell (Fritsch, 1959), within a single species. The stained centroplasm may have a rodlike, reticulate or granular appearance, not similar to that seen in the chromosomes or other nuclear constituents of higher organisms, either as viewed with the light microscope (Fritsch, 1959) or with the electron microscope (Ris and Singh, 1961). However, according to Ris and Singh (1961) ribosomes appear to be present in both the centroplasm and the chromatoplasm. Otherwise, the centroplasm shows little organized structure (Ris and Singh, 1961; Hopwood and Glauert, 1960). At cell division, which occurs by annular constriction, the centroplasm is generally divided into two, more or less equal, daughter regions, one located in each new cell.

The cortical chromatoplasm, as its name indicates, contains the pigment, the blue-green phycocyanin, which characterizes most of the Cyanophyceae. Other pigments include chlorophyll a, carotenes, xanthophylls, and a phycoerythrin (Fritsch, 1959; Geitler, 1960). The chromatoplasm of these cells also contains presumed storage products such as glycogen and cyanophycin, which is believed to be a proteinaceous substance. This latter substance disappears when cells are placed in the dark.

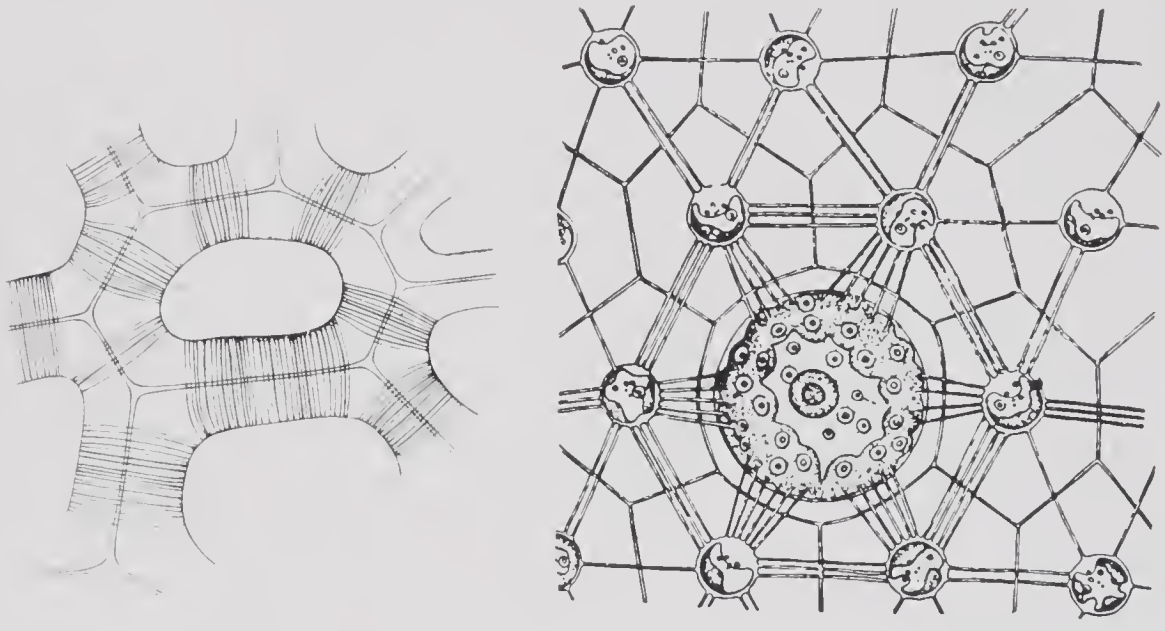
In the electron microscope, as shown in Figs. 6 and 7, the chromatoplasm is seen to be filled with a complex lamellar system (Geitler, 1960; Hall and Claus, 1962; Lefort, 1960a, b; Menke, 1961; Ris and Singh, 1961; Shatkin, 1960). This membranous system is comprised of 140 A.-thick lamellae penetrating the cytoplasm. The lamellae are paired unit membranes, sometimes separated by an electron transparent region of vesicular appearance (Ris and Singh, 1961). According to Shatkin (1960), all the chlorophyll and carotenoids, as well as the photophosphorylating activity, of fractionated cells are found associated with these membranes and apparently bound to them.

III. Special Structures

A. *Plasmodesmata*

1. Distribution and Morphology

Credit for the discovery of the fine protoplasmic strands between the cells of plants is generally given to E. Tangl. In 1879 he described the presence of these threads, or *plasmodesmata* as Strasburger (1901) named them, between the cells of the endosperm of species of *Strychnos, Phoenix,* and *Areca* (Fig. 8a). Tangl also pointed out the protoplasmic



a b

Fig. 8. (a) Iodine-stained endosperm of *Strychnos nux-vomica,* showing plasmodesmata between a cell and six of its neighbors. From Tangl (1879). (b) Cells of colonial *Volvox* showing plasmodesmatal connections. From Fritsch (1935).

nature of the strands. Furthermore, he clearly recognized the importance of these structures in intercellular movement of storage products and other metabolites and described the plasmodesmata as an "open system between the cells." To be sure, earlier workers had noted striations across the cell wall, as has been pointed out by Strasburger (1901) and by Meyer (1920). But, Tangl was the first cytologist to recognize what he saw.

Following Tangl's observations, numerous other reports of plasmodesmata were published, and Strasburger (1882) and Kienitz-Gerloff (1891b) suggested that protoplasmic connections were to be found between all the cells of a plant. Their generalization subsequently has found ample support in many studies of various plant tissues and organs,

as viewed both with the light and electron microscopes. In a few cases, including species from quite different groups of plants, it has been possible to carry out systematic examination of entire plants and to demonstrate plasmodesmata in all tissues. For example, Kuhla (1900) studied the European mistletoe, a dicotyledonous plant, and found that "all of the living cells of *Viscum album* are united to one another through plasma connections." Livingston (1935) noted that plasmodesmata were to be found throughout the tobacco plant. Gardiner and Hill (1901) made a detailed study of the tissues of pine trees (*Pinus sylvestris* and *P. pinea*) and came to similar conclusions. Meyer (1896, 1920) found plasmodesmata throughout the spherical green alga, *Volvox* (Fig. 8b).

Although plasmodesmata have been studied chiefly in higher plants (they are also common in animals, cf. review of Meyer, 1920), their presence is nearly ubiquitous in multicellular plants including mosses, liverworts, gymnosperms, and many algae (members of the Chlorophyceae, Rhodophyceae, and Phaeophyceae) as well as the flowering plants (Fritsch, 1935, 1959; Kienitz-Gerloff, 1891a, b; Meeuse, 1957; Meyer, 1920; Mühldorf, 1937; Strasburger, 1901). Doubt was cast upon the validity of some of the earlier studies because of the techniques employed to demonstrate plasmodesmata. However, Mühldorf (1937), in an exhaustive study, compared the various methods of demonstrating plasmodesmata. He then examined about 150 representatives of diverse groups of plants, again successfully demonstrating the presence of plasmodesmata.

The fine, threadlike plasmodesmata are barely visible in the light microscope, and for this reason they customarily were stained and caused to swell or expand in solutions containing iodine and sulfuric acid, or similar reagents. Following such treatment, the plasmodesmata may be seen fairly readily, especially in thick-walled tissues. The number of plasmodesmata seen between cells is variable. Kuhla (1900) found from 0.5 to 50.0 per 100 μ^2 in various tissues of *Viscum* (average ca. 500 per cell) and similar numbers (1.5–40.8 per 100 μ^2) were found in tissues of tobacco by Livingston (1935). In meristematic cells of onion (*Allium cepa*) and *Elodea*, Strugger (1957a, b), in an electron microscope examination, counted 6–7 plasmodesmata per μ^2, or a total of 10,000–20,000 per cell.

The plasmodesmata in all land plants are unforked cylinders (Meeuse, 1957; Meyer, 1920; Mühldorf, 1937; Strasburger, 1882) which penetrate the cell wall perpendicular to its surface. Thus, the channels through which the plasmodesmata pass are generally the shortest routes between adjacent cells. The plasmodesmata may be randomly distributed

in the cell wall or they may be collected together in groups within depressions, or narrow regions of the wall, known as pits (Fig. 9a).

Occasional workers, such as Jungers (1930, 1933), have reported that branched or forked plasmodesmata occur. However, such claims have generally resulted from observations of cytological artifacts or from partial superposition of two plasmodesmata and have not been supported in reinvestigation by other workers (Livingston, 1935; Mühldorf, 1937; Meeuse, 1957). Similarly, the reports by Huber *et al.* (1956) of forked plasmodesmata, as seen in the electron microscope, are very probably in error. The supposed plasmodesmata in their Fig. 16 appear to be many times greater in diameter than those generally reported. It is clear from the figures of these investigators that the sections examined were badly damaged during preparation. The structures shown look more like small folds in the sections than like plasmodesmata; these irregularities are perhaps compression artifacts from microtome sectioning or are due to differential swelling of the cell wall (cf. also the pertinent discussion by Sitte, 1958, Fig. 5 and pp. 460–461).

With few exceptions (see below), cytologists have agreed that plasmodesmata are seen only between cells sharing a common wall. In other words, they are not generally seen at the outer walls of epidermal cells (Fig. 9a) or at parts of cells adjacent to intercellular spaces. This has long been known from classical studies (Strasburger, 1901; Mühldorf, 1937; Meeuse, 1957) and has been confirmed in the electron microscope for intercellular spaces by the studies of Strugger (1957a). The outer cell walls shown in the electron micrographs of Roelofsen and Houwink (1951), O'Kelley and Carr (1953), and Schieferstein and Loomis (1959) also appear to lack plasmodesmatal pores. However, Scott and her coworkers (Scott *et al.*, 1948, 1958) have reported that cytoplasmic strands are visible in the outer walls of epidermal cells when viewed in light microscope preparations (Fig. 9b). These researchers have provided some supporting evidence from electron microscope studies (Scott *et al.*, 1958). However, they examined cell walls prepared by ultrasonic vibration. It is not entirely clear how they distinguished outer epidermal cell walls from inner ones, since the outer cuticle was apparently removed in preparation, as has been pointed out by Schieferstein and Loomis (1959). Claims by Huber *et al.* (1956), Hassenkamp and Liese (1957), Schumacher (1957), Beer and Setterfield (1958), and Schnepf (1959) of electron microscope observations of plasmodesmata-like threads, or ectodesmata, in thin sections of outer walls are quite unconvincing. It is interesting to note that there is disagreement even among those who have reported these structures, inasmuch as Schnepf (1959) ques-

tions the interpretation of the electron micrographs made by Scott *et al.* (1958). In any case, the possibility of the presence of plasmodesmata-like threads in the outer wall of epidermal cells, especially as postulated by Schumacher and his colleagues in Germany (Schumacher and Halbsguth, 1938; Schumacher, 1942, 1957; Lambertz, 1954; Schumacher and Lambertz, 1956; Schnepf, 1959; Sievers, 1959, 1960; Franke, 1960a, b, 1961) is very interesting. It is to be hoped that better electron microscope evidence will be presented than has thus far been provided.

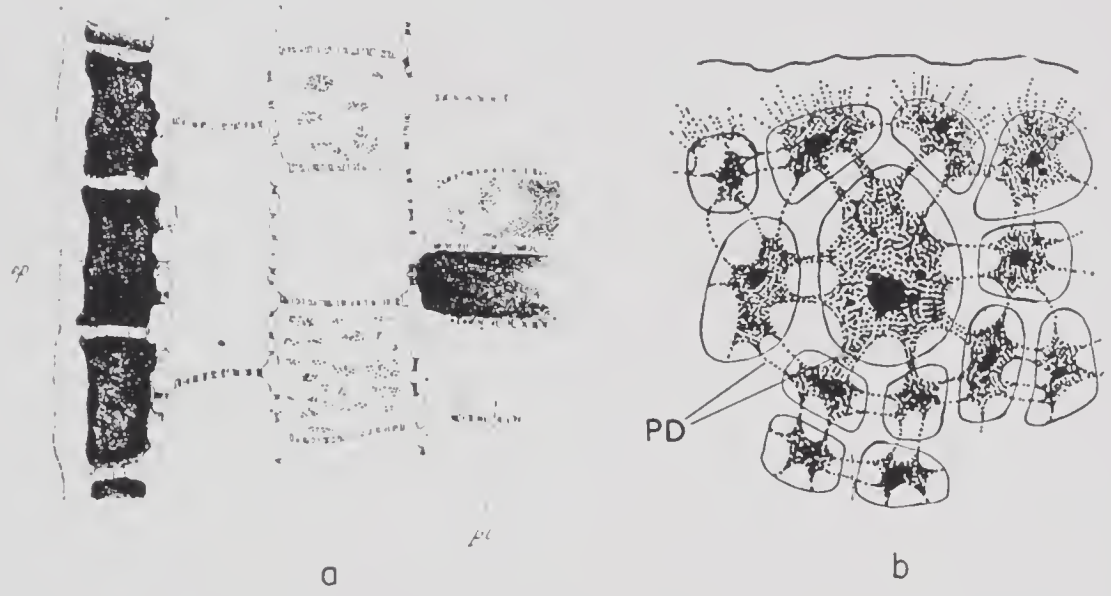


FIG. 9. (a) Longitudinal, radial section of the lower side of a cotyledon from *Pinus pinea*, showing plasmodesmata between cells. Cytoplasmic strands are absent from the outer walls of the epidermal cells (*ep.*). Note that many of the plasmodesmata are grouped together in "pits," depressions in the cell wall. From Gardiner and Hill (1901). (b) Transverse section through a portion of an orange (*Citrus sinensis*) leaf, showing plasmodesmata (*PD*) between cells and cytoplasmic strands in the outer walls of epidermal cells. Tissue treated with iodine-potassium iodide (IKI) and sulfuric acid. From Scott *et al.* (1948).

Measurement of the size of the plasmodesmata has been based upon occasional favorable materials in which unaltered bridges could be seen. For example, Livingston and Bailey (1946) examined living cells of the cambium of pine (*Pinus strobus*) and the ray cells of redwood (*Sequoia sempervirens*), as well as other plants, and reported the plasmodesmata to be about 0.2 μ in diameter. In addition, preparations of cell walls, as viewed in the electron microscope, reveal that the walls are riddled with pores about 0.1 μ in diameter (Meeuse, 1957; Roelofsen and Houwink, 1951; Scott *et al.*, 1956, 1958; Mühlethaler, 1961; Dawes *et al.*, 1961; Huber *et al.*, 1956). These pores are believed to be the plasmodesmatal pores. Recently, many electron micrographs of "ultrathin"

sections of plant cells have been published and from these it has been possible to calculate the size of the protoplasmic threads as well as that of the pores. Strugger (1957a, c) made a careful study of onion (*Allium cepa*) cells and found that although the size of the wall pores was about 1100–1900 A. (ca. 0.1–0.2 μ) in diameter, the cytoplasmic thread was only about 300–400 A. thick. Similarly, other investigators' electron micrographs indicate a diameter of about 0.05–0.1 μ, frequently even smaller, in diverse plants (Heitz, 1957; Buvat, 1957a; Perner, 1958; Whaley *et al.*, 1960; Poux, 1961). However, Strugger (1957c) pointed out that substantial shrinkage occurs during preparation of the tissues for the electron microscope. This would lead to a decrease in the diameter of the cytoplasmic thread. Moreover, contraction of the thread would also result from the tension placed upon it by shrinkage of the connected protoplasts on each side of the cell wall. Strugger believes that the cytoplasmic connections of living cells fill the pores completely and therefore would be about 0.1–0.2 μ in thickness.

2. *Cytoplasmic Nature*

The cytoplasmic nature of the plasmodesmata was recognized by Tangl (1879), who observed that plasmodesmata and the cytoplasm stained quite similarly, but that each stained differently from the cell wall. Subsequent observers (Strasburger, 1882, 1901; Meyer, 1920; Mühldorf, 1937; cf. also Meeuse, 1957) found similar staining properties with various other dyes. Electron micrographic studies also have confirmed the cytoplasmic nature of the plasmodesmata (Strugger, 1957a; Buvat, 1957a; Whaley *et al.*, 1960). Strugger (1957a) and Buvat (1957a, 1960) reported that the cell membranes, or plasmalemmae, of adjacent cells are continuous with each other across the plasmodesmatal canals, but their published figures are not entirely convincing. However, Fig. 10 and also the pictures of Whaley *et al.* (1960) show this continuity and leave little doubt of its existence.

The cytoplasmic continuity between cells has been supported by various indirect evidence as well. In part, viral movement in plants occurs through the plasmodesmata and it is clear that viruses do not move across grafts until tissue union has occurred (Bennett, 1943; Esau, 1948). It is now recognized that some viruses move through the phloem tissues, whereas others probably diffuse through the plasmodesmata (Bennett, 1943; Crafts, 1951; Esau, 1948; Livingston, 1935).

Bennett (1943) studied the rate at which various viruses passed across freshly made graft unions in Turkish tobacco plants (*Nicotiana tabacum*). He found that ringspot and cucumber viruses from infected

scions of tobacco led to contamination of the stock within 2 days. Presumably this occurred because the viruses passed through the protoplasmic connections, which were known to become established in the same period. In contrast, curly-top virus required a longer period,

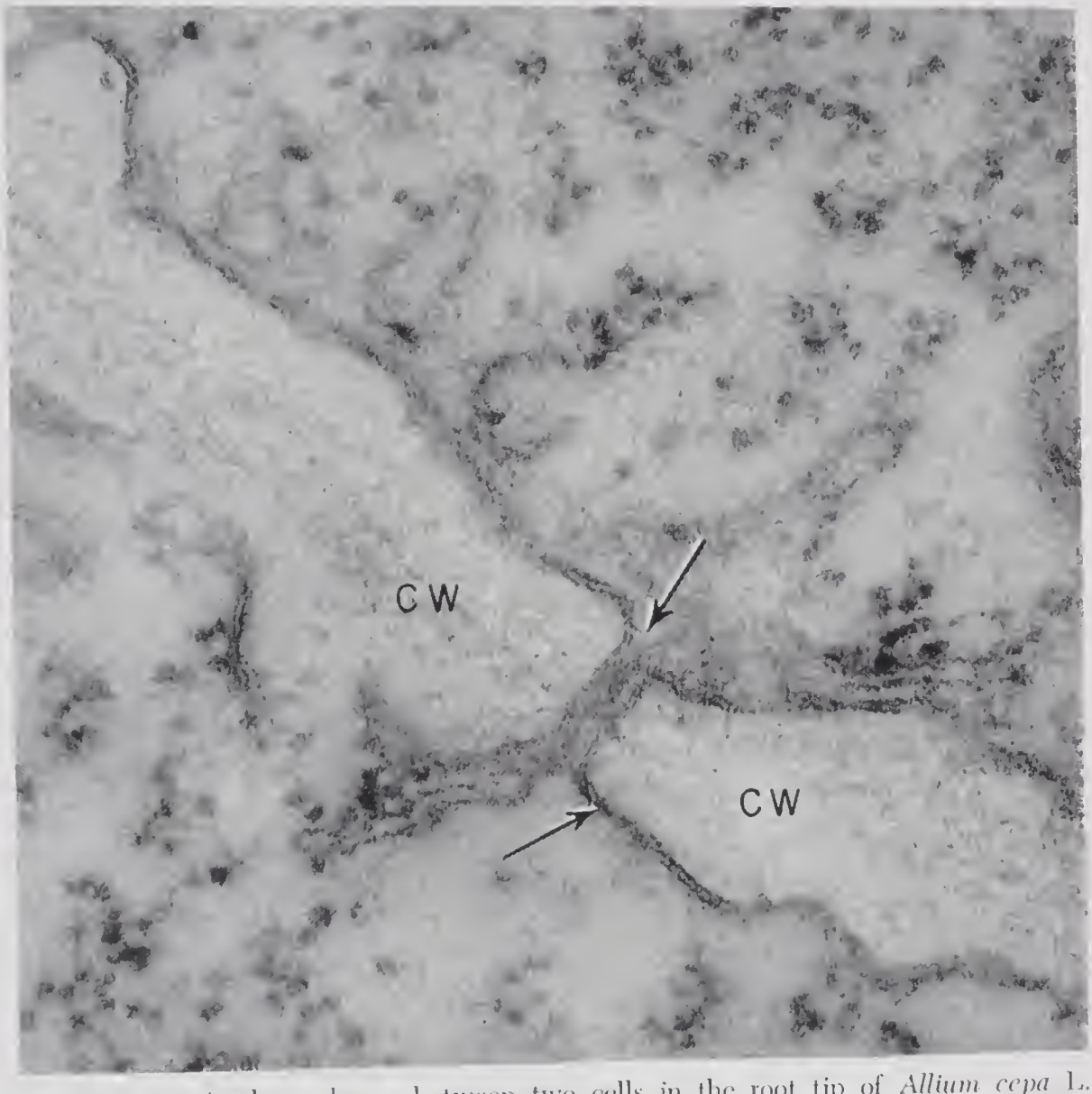


FIG. 10. A plasmodesma between two cells in the root tip of *Allium cepa* L. The cell membranes, or plasmalemmae (arrows), of the two cells are seen to be in continuity with each other across the plasmodesmal canal. Note also the cytoplasmic ribosomes, shown here at high magnification. The cell wall is marked *CW*. Magnification: × 130,000. From Machado and Porter (unpublished).

apparently dependent upon the establishment of phloem connections, before infection resulted.

Although viruses have never been observed within plasmodesmata, it is clear from the size of these particles (Table I) that it may be possible for them to move through the canals (cf., also Esau, 1961;

Shalla, 1959). The one exception is beet-yellows virus (possibly also PVX and TMV), which would have to be properly oriented, or folded, in order to pass through a plasmodesma, even if the virus were to pass through without its protein coat. By means of the recent heavy-metal staining techniques and by means of the excellent sections which can be

TABLE I
DIMENSIONS OF PLANT VIRUSES[a]

Virus	Diameter (A.)	Length (A.)
Tobacco mosaic (TMV)	180	3,000
Potato virus	100	5,000
Beet yellows	100	10,000
Tomato bushy stunt	300 ± 10[c]	
Turnip yellows	280 ± 10[c]	
Southern bean mosaic	280 ± 10[c]	
Tobacco ringspot	280 ± 20[c]	
Polio[b]	280[c]	

[a] After Klug and Caspar (1960).
[b] Animal virus, included for the purpose of comparison.
[c] Spherical viruses.

made with diamond microtome knives, it should be possible to demonstrate both the presence of viruses within and their movement through the plasmodesmata.

3. *Developmental Origin*

The origin of the plasmodesmata remains obscure. Their presence has been observed in the meristematic tissues of many species, but the process of their initiation has not been clearly seen. Cell-plate formation in cell division has long been studied in the hope that the origin of plasmodesmata might be viewed (cf. reviews by Kienitz-Gerloff, 1891c; Meyer, 1920; Strasburger, 1901). In the past such efforts have been more or less unsuccessful, as pointed out by the authors just cited. However, recent electron microscope studies of cell-plate formation have again led various workers to relate plasmodesmata formation to events at cell-plate formation. Buvat and Puissant (1958) and Porter and Machado (1960), for example, hint that elements of the endoplasmic reticulum which traverse the region of the forming cell plate might be important in the origin of plasmodesmata. Indeed, a number of cytologists have suggested elements of the reticulum to be present in differentiated plasmodesmata (e.g., Buvat, 1960; Poux, 1961; Whaley *et al.*, 1960; cf. also Manton, 1961). It is true that elements of the endoplasmic

reticulum are very commonly in close proximity to the plasmodesmata (Figs. 2, 3, 14, and 15) and plasmodesmata do contain a fine "pluglike" structure (Fig. 10), as indicated by Porter and Machado (1960). Yet not one of the numerous published electron micrographs of plasmodesmata shows convincing evidence of the presence *within* the plasmodesmata of elements of the endoplasmic reticulum. Nor were Porter and Machado (1960), in their thorough study of meristematic cells of onion roots, able to confirm the view that differentiated plasmodesmata ever contain elements of the endoplasmic reticulum.

An interesting alternative view (albeit not necessarily incompatible with that mentioned above), tentatively suggested by Buvat and Puissant (1958) and Porter and Machado (1960), is that the plasmodesmata are formed as simple interruptions or discontinuities in the cell plate, when the plate is constituted from the "pectin" vesicles.

Although the origin of plasmodesmata may in part be found in the presence of elements of endoplasmic reticulum stretched across the developing cell plate or through discontinuities in the forming cell plate, events at cell division cannot account for the origin of *all* plasmodesmata. There are, for instance, several tissues in which cell walls are traversed by plasmodesmata which do not appear to have been associated with cell division. For example, plasmodesmata are formed in the walls of the invasive, penetrating haustoria of the parasitic flowering plant, dodder (*Cuscuta*), adjacent to the cells of its host plants (Bennett, 1944; Esau, 1948, 1953; Schumacher and Halbsguth, 1938), although earlier students (e.g., Kienitz-Gerloff, 1891d; Strasburger, 1901) were unable to find them. The tissues of other parasites, such as *Orobanche* (Schumacher and Halbsguth, 1938) and European mistletoe (*Viscum album*) (Esau, 1948, 1953) also establish protoplasmic continuity with their host plants, although, again, Kienitz-Gerloff (1891d), Kuhla (1900), and Strasburger (1901) said they did not find plasmodesmata. Furthermore, the plasmodesmatal connections between the tissues of grafted hybrids (Strasburger, 1901; Bennett, 1943; Bálint, 1910) and in graft chimeras (Hume, 1913) cannot have arisen through any processes directly linked with cell division.

Strong supporting evidence for a similar conclusion comes from yet another source, as long ago pointed out by Strasburger (1901) (cf. also Meyer, 1920). The epidermal layer of diverse plants is known to divide anticlinally. In other words, cell division in the epidermis leads to an increase in number of cells *only within* the single-layered epidermis. Such division increases the length and girth of this layer (e.g., around a shoot) but *not* its thickness. By treating seeds with colchicine, for

example, it has been possible to produce shoot apices having polyploid epidermal cells, whereas all the other cells of the shoot apex were diploid. In such chimeras, the single layer of epidermal cells, and only those, were subsequently found to be polyploid throughout the mature organs of the plant (Satina *et al.*, 1940; Satina and Blakeslee, 1941). The *inner* (tangential), longitudinal walls of the epidermis do not arise through cell division, except in seed embryos, but increase in size through elongation of existing walls. Despite this very different mode of wall origin, the walls are traversed by many plasmodesmata. Kuhla's (1900) data, as well as those of Kienitz-Gerloff (1891b), indicate that the number of plasmodesmata across the longitudinal walls is approximately the same as found through the transverse end walls and the walls of subepidermal cells, although in tobacco fewer plasmodesmata are present in the tangential wall than in the radial and end walls (Livingston, 1935). In each of these examples as well as in many others, it is quite clear that plasmodesmata arise in *already existing* epidermal cell walls.

In addition to the examples already cited, it seems highly probable that plasmodesmata are formed in the existing cell walls of fiber initials. These cells grow by "apical intrusion" and it is known that paired pits arise between the invading tip of the fiber initials and adjacent cells (Bannan, 1950; Esau, 1953). Thus, it seems almost certain that these pits contain plasmodesmata, although apparently no one has looked for them.

In summary, the presence of plasmodesmata between cells of graft hybrids, between parasites such as dodder and mistletoe and their hosts, between epidermal and subepidermal cells of many higher plants, and between stationary cells and the invasive tips of fiber cells provides excellent experimental material for electron microscopic study of the formation of plasmodesmata. In each case it should be possible to determine the developmental course leading to the cytoplasmic connection of adjacent cells through *existing walls* and to compare this process with the origin of plasmodesmata in walls formed at *cell division*.

4. Function

Despite the universal acceptance of the physiological importance of the plasmodesmata, there are relatively few well established evidences of their role. It is commonly thought that their chief functions are related to the conduction of metabolites from one region of the plant to another and the movement of hormonal stimuli important in growth regulation. However, in vascular plants studies of the movement of

various tracers, including radioactive compounds, indicate that rapid, long-distance movement of metabolites occurs chiefly in the phloem tissue (cf. Esau, 1961; Esau *et al.*, 1957; Schumacher, 1930). In nonvascular tissues such as those found in many mosses, liverworts, and fern gametophytes, and in vascularized plants within regions comparatively isolated from phloem sieve cells, it seems very probable that the plasmodesmata are of particular importance. It is well known from plasmolytic and microsurgical studies that the plasmalemma, or cell membrane, is impermeable to many solutes. Thus, the plasmodesmata would provide a means of intercellular movement for such solutes. Furthermore, since it seems certain that localized movement of various viruses occurs via the plasmodesmata, it seems reasonable to assume that other substances do so as well.

The gametophytes of ferns show many distinct growth correlations which seem almost certainly to be effected through the cytoplasmic connections. For example, Döpp (1959) demonstrated that an inhibitor produced by the meristem of a fern gametophyte prevented formation of antheridia upon cells of the thallus. If the meristem was destroyed, or pieces of the gametophyte partially or completely surgically isolated from the region of the thallus bearing the meristem, antheridia were produced. All evidence points toward conduction of an antheridium-inhibiting stimulus through the plasmodesmata.

Townsend (1897) carried out very interesting work on filaments of cells; he employed moss protonemata, fern gametophytes, and hairs from the leaves and stems of flowering plants. Townsend observed that the nucleus in one cell could induce experimentally enucleated cytoplasm in adjacent cells to re-form a plasma membrane if the protoplasmic connections between the two cells were intact. In contrast, enucleated protoplast with severed plasmodesmata and isolated, enucleated protoplasts formed no cell membranes. Thus, Townsend elegantly demonstrated that the nucleus was required for formation of a complete cell membrane and that the stimulus supplied by the nucleus was transmitted via the plasmodesmata.

In studies of the movement of the "flowering stimulus" induced by appropriate day-length, it has been reported that the stimulus moves from the induced plant across graft unions to noninduced plants within 4 days (Roberts, 1949). The induction stimulus probably occurs through newly formed plasmodesmata, since, unlike plasmodesmatal connection, formation of phloem connection at grafts usually requires a period longer than 4 days.

The *lateral* conduction of the growth stimuli which result in the

geotropic growth of plants may possibly occur through plasmodesmata. In the oat coleoptile, for example, the cylindrical leaf has two narrow, longitudinal vascular bundles, but no transverse vascular tissue. Most of the body of the leaf comprises parenchymatous cells. When such a leaf is placed horizontally on its side, it grows more rapidly on its lower side than on its upper, until the leaf is again vertical. Accumulation of a growth-promoting hormone at the lower side of the horizontal organ has been shown to be the basis of such geotropic phenomena (cf., Went and Thimann, 1937). It seems possible that the hormone is conducted across the boundaries of adjacent parenchymatous cells through the plasmodesmata.

These several examples of intercellular relationships, which are only a few of the great number which might be cited, are all conveniently interpreted in terms of the presence of the plasmodesmata. There is little reason to believe that they should be interpreted in any other manner. However, it must be fully recognized that at present there is no *direct* experimental proof of the physiological role of the plasmodesmata in any of these phenomena. Clearly, such an important assumption as the role played by these protoplasmic bridges should be placed upon a sounder basis. This should be possible to do by carrying out physiological studies in conjunction with the autoradiographic techniques which now may be used for study of tissues with both the electron and light microscopes.

The possibility of the function of ectodesmata, plasmodesmata found in outer cell walls, in such processes as uptake of nutrients from outside the aerial portions of the plant, environmental perception, cuticular transpiration, foliar excretion, etc., has been suggested by Schumacher and his colleagues (cf. Meeuse, 1957; Franke, 1961). However, little physiological evidence of a compelling nature has been presented in favor of these views. Furthermore, most other workers have failed to confirm the cytological observations underlying the possible importance of ectodesmata. Nonetheless, the significance of such small canals, if they could be adequately demonstrated, would probably be great. For this reason, as with the role of plasmodesmata in general, it is to be hoped that careful electron microscope studies and appropriate tracer studies will presently help to resolve the current uncertainty as to the nature and possible function of ectodesmata.

B. Dictyosomes

Shortly after the publication of Golgi's (1898) and Holmgren's (1901) famous papers on the structure of a cytoplasmic, reticular system,

Bensley (1910) observed fine vacuolar, canal-like networks in the meristematic cells of lily, iris, and onion roots. Moreover, his studies led him to believe that as the roots matured, the networks *differentiated into* the familiar vacuoles of mature plant cells. On the basis of these observations, Bensley decided that the vacuolar system of plants was the physiological and morphological equivalent of the Golgi apparatus of animal cells. Following Bensley's studies, observations of Golgi networks were made by many other plant cytologists (cf. Nevins, 1933; Zirkle, 1937). Guilliermond and Mangenot (1922), Scott (1929), and Bose (1927) showed in a variety of plants that the *so-called* Golgi apparatus was indeed the network of small, filiform vacuoles which characterize meristematic cells of plants. These workers, also, homologized this vacuole network, and its derivative in mature cells, with the Golgi apparatus of animal cells. Although their cytological observations were doubtless correct, it is now generally agreed that the homology they postulated was not based on adequate grounds and that there is no satisfactory evidence of any relationship between the vacuolar system and Golgi apparatus (cf. Beams and King, 1935; Bowen, 1927; Geitler, 1955; Jones, 1938; Küster, 1935; also the entire body of electron microscope evidence, discussed below).

Bowen (1927), who had substantial experience with the platelike Golgi bodies, or dictyosomes, of animals, also examined plant cells. He was able to demonstrate that osmophilic platelets, which were very similar to those of animal cells, could also be discerned in cells from kidney bean roots and the spermatazoids of mosses. The osmophilic platelets (Fig. 11) were densely stained at the margins of the disks and more lightly in the centers. When viewed in profile, they appeared to be rod-shaped or slightly curved. These structures did not correspond in their various properties to any previously described components of plant cells (e.g., mitochondria, vacuoles, plastids) but were, according to Bowen, homologous with the Golgi platelets of animal cells. These observations were confirmed and extended through the careful investigations of various subsequent workers (Beams and King, 1935; Jones, 1938; Nevins, 1933; Patten *et al.*, 1928). Furthermore, the distinctiveness of these platelets was demonstrated by the results of studies in which the shoots of *Elodea*, the roots of wheat (*Triticum vulgare*) (Jones, 1938), and the root tips of bean (*Phaseolus vulgaris*) (Beams and King, 1935) were ultracentrifuged. In tissues subjected to such treatment, the protoplasmic organelles separated into layers of different density within the cells; the osmophilic platelets were in well-defined layers, distinct from those which contained vacuoles, mitochondria, etc. (Fig. 12).

Furthermore, the platelets did not take up stains regarded as specific for vacuoles or mitochondria.

On the basis of the size, form, number, and staining properties of the osmophilic platelets, in addition to their similarity to the structures

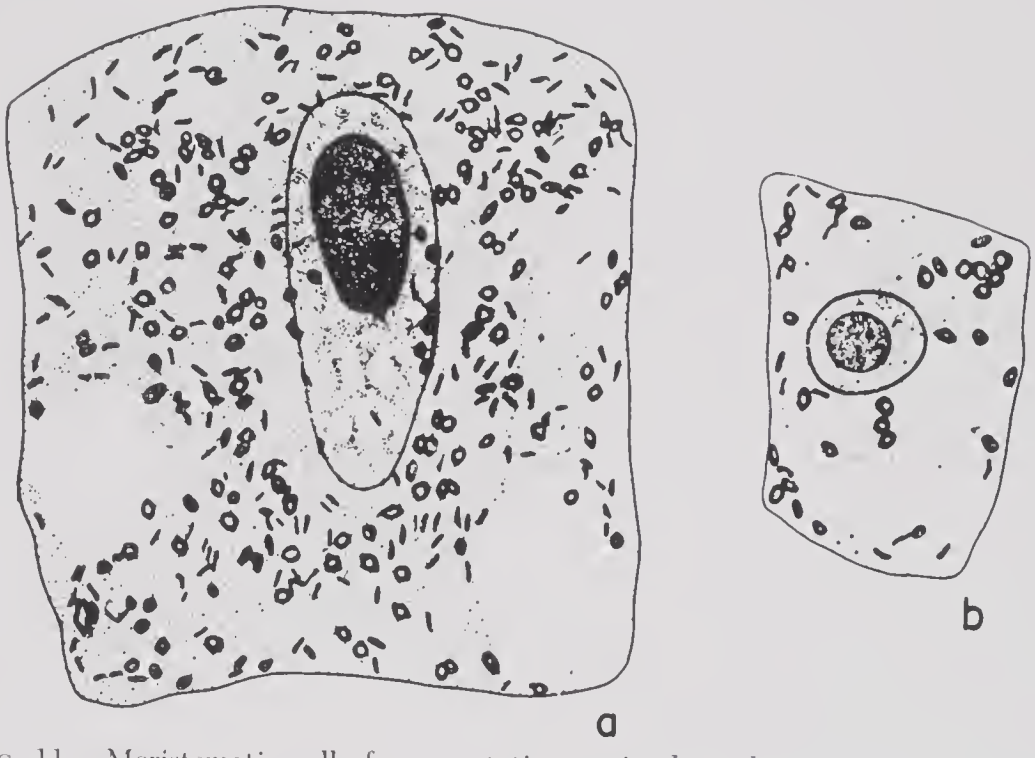


FIG. 11. Meristematic cells from root tips, stained to show osmophilic platelets. (a) Large cell from the central core of a barley root tip. (b) Cell from the root tip of a kidney bean plant. From Bowen (1927).

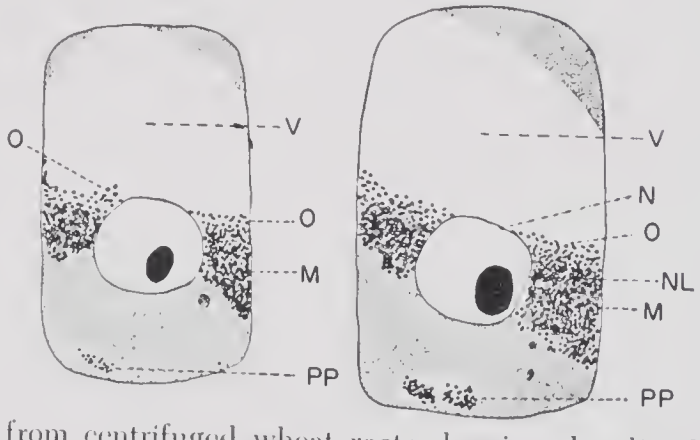


FIG. 12. Cells from centrifuged wheat roots showing the distribution of the cell contents: Mitochondria (*M*), nucleus (*N*), nucleolus (*NL*), osmophilic platelets (*O*), proplastids (*PP*), and vacuole (*V*). From Jones (1938).

seen in animal cells, it seems highly probable that the disks observed in plants by Bowen are identical to the dictyosomes (= Golgi bodies) now seen in the electron microscope and described below. These dictyosomes have been observed by means of the electron microscope in repre-

TABLE II

ELECTRON MICROSCOPE CITATIONS OF DICTYOSOMES IN PLANTS

Plants	References[a]
Flowering plants	
Monocotyledons	
Allium cepa	(*4, 5, 7, 9, 10, 11, 12, 21, 26, 35, 36, 40*)
Chlorophytum	(*27*)
Elodea	(*6, 7, 22*)
Trianea (*Limnobium*)	(*27*)
Zea mays	(*25, 26, 27, 42, 43*)
Diverse other grasses	(*7, 8, 10, 16, 37*)
Dicotyledons	
Antirrhinum	(*15*)
Chrysanthemum	(*7, 12, 23*)
Cucurbita pepo	(*27*)
Lens culinaris	(*7*)
Oenothera	(*21*)
Parkinsonia	(*24*)
Phajus	(*7*)
Pisum sativum	(*41*)
Tradescantia	(*1*)
Vicia faba	(*27*)
Ferns	
Pteridium aquilinum (bracken)	(*18*)
Mosses and liverworts	
Anthoceratales, 1 genus	(*14, 31*)
Bryales, 5 genera	(*18*)
Jungermaniales, 4 genera	(*18*)
Marchantiales, 2 genera	(*18*)
Sphagnales, 1 genus	(*18, 29*)
Algae	
Green	
Chlamydomonas	(*38*)
Chrysochromulina	(*32*)
Euglena	(*13*)
Micromonas	(*33, 39*)
Nitella	(*16, 17*)
Nitellopsis	(*34*)
Pedinomonas	(*33*)
Red	
Lomentarium	(*2*)
Porphyridium	(*3*)
Brown	
Dictyota	(*30*)
Scytosiphon	(*28*)
Yellow	
Vaucheria	(*19, 20*)

[a] *References*: (*1*) Bal and De (1961); (*2*) Bouck (1962); (*3*) Brody and

sentatives of virtually all the major groups of plants, including many monocotyledons and dicotyledons, a fern, all the chief groups of mosses and liverworts, and in many diverse algae, as reported in the approximately fifty papers listed in Table II. In the main, observations in the flowering plants have been made upon root meristems. However, the dictyosomes do not appear to be significantly different in those shoot apices, embryos, and leaves which have been examined. Thus, there seems to be a basic similarity in the appearance of dictyosomes from different organs and even from taxonomically distant groups of plants.

Although the plant dictyosomes are often somewhat simpler in appearance than the Golgi bodies of animal cells, the resemblance of the two is quite striking. In general, as illustrated in Fig. 13 (also, see Fig. 1), transverse sections of the dictyosomes reveal somewhat U-shaped (especially after permanganate fixation) or flat groups of about six double-membraned lamellae or cisternae. Each lamella is about 0.4–1.5 μ long by about 200 A. thick and typically has terminal inflations or bubble-like dilations, although these are frequently absent.

The two membranes of a lamella are quite electron dense and about 70 A. thick. In size and form they resemble those of the endoplasmic reticulum (Fig. 13), but at present there is no convincing evidence of an actual relationship between the dictyosomes and endoplasmic reticulum or any other organelles. Moreover, in both plants and animals, the dictyosomes are always devoid of ribosomes, in contrast with the endoplasmic reticulum (Fig. 1).

In many cells, the dilated ends of the dictyosome lamellae appear to be liberated into the cytoplasm, where they are seen as small vesicle-like structures. In the root cap of onion cells, the freed vesicles *seem* to move away from the dictyosome to the plasma membrane, at the

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Vatter (1959); (*4*) Buvat (1957b); (*5*) Buvat (1957c); (*6*) Buvat (1958a); (*7*) Buvat (1958b); (*8*) Buvat (1959); (*9*) Buvat and Carasso (1957); (*10*) Buvat and Mousseau (1960); (*11*) Genèves (1960); (*12*) Genèves *et al.* (1958); (*13*) Gibbs (1960); (*14*) Chardard (1957); (*15*) Heitz (1957); (*16*) Hodge (1956); (*17*) Hodge *et al.* (1956); (*18*) Heitz (1958); (*19*) Greenwood (1959); (*20*) Greenwood *et al.* (1957); (*21*) Menke (1960a); (*22*) Menke (1960b); (*23*) Lance (1957); (*24*) Larson and Lewis (1961); (*25*) Lund *et al.* (1958); (*26*) Mollenhauer *et al.* (1961); (*27*) Perner (1958); (*28*) Manton (1957a); (*29*) Manton (1957b); (*30*) Manton (1959); (*31*) Manton (1960); (*32*) Parke *et al.* (1959); (*33*) Manton and Parke (1960); (*34*) Klima and Jarosch (1961); (*35*) Porter (1957); (*36*) Porter and Machado (1960); (*37*) Poux (1961); (*38*) Sager and Palade (1957); (*39*) Rouiller and Fauré-Fremiet (1958); (*40*) Strugger (1957d); (*41*) Sitte (1958); (*42*) Whaley *et al.* (1959a); (*43*) Whaley *et al.* (1959b).

same time becoming more electron dense. They appear to fuse with the plasma membrane and to release their contents outside the membrane, next to the wall (Mollenhauer *et al.*, 1961).

In oblique and planar sections, as shown in Figs. 14 and 15, the lamellae of the dictyosomes are seen to be flat and roughly circular in

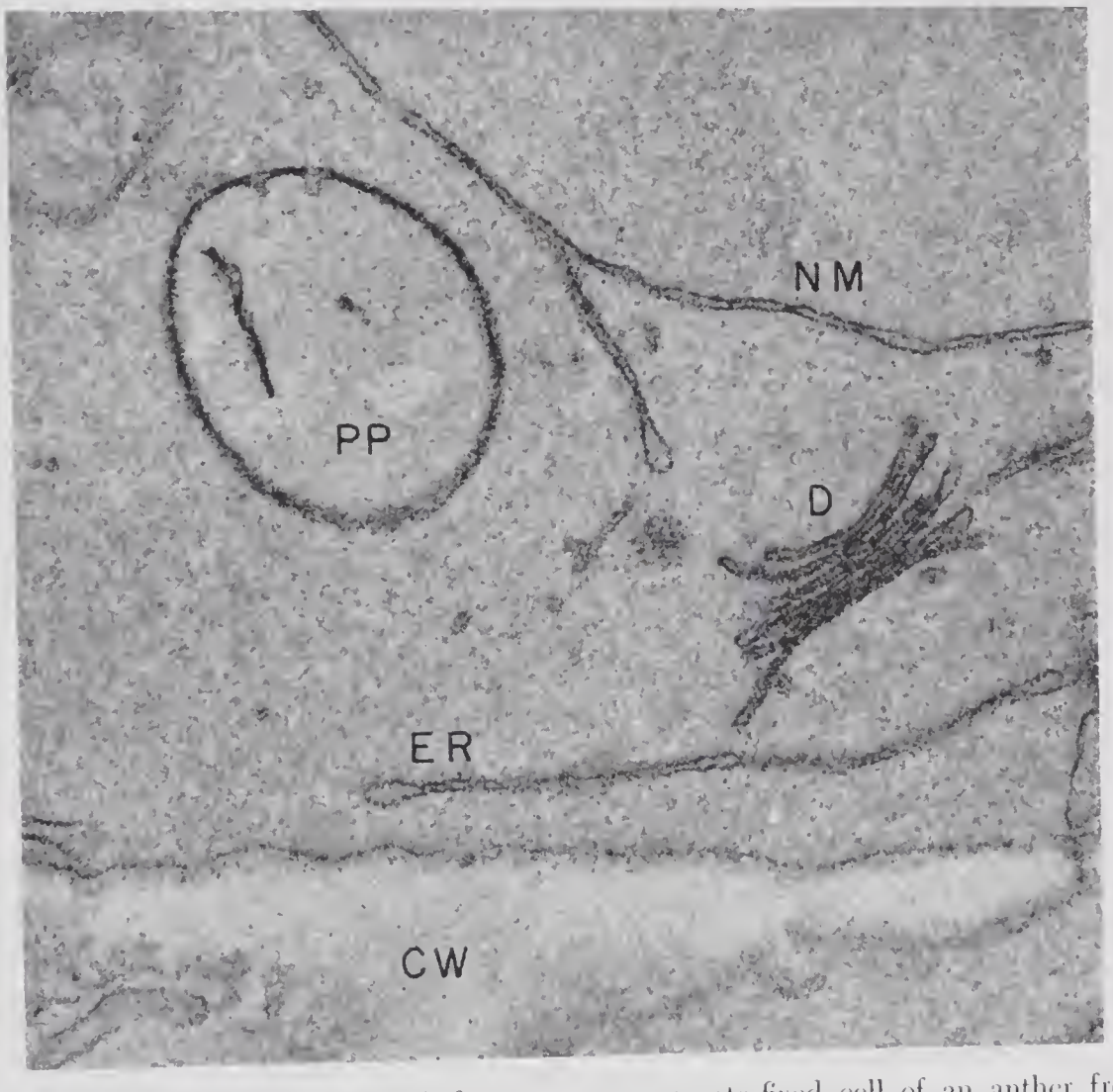

FIG. 13. A dictyosome (*D*) from a permanganate-fixed cell of an anther from *S. ionantha*. Six double-membraned lamellae, characterizing the dictyosomes of many plants, are shown. A cell wall (*CW*), endoplasmic reticulum (*ER*), a nuclear membrane (NM), and a proplastid (*PP*) are also present. Magnification: × 43,000. (Electron micrograph by Ledbetter and Porter.)

outline and often to have vesicular, radiating arms (see also Bouck, 1962, Fig. 17; Hodge *et al.*, 1956, Figs. 3 and 4; Manton, 1960, Figs. 2–4, and 7; Perner, 1958, Figs. 15-17; and Whaley *et al.*, 1960, Fig. 7).

The number of dictyosomes per cell seems often to be large. Sitte (1958) estimated that there were well over 30 per cell in bean roots. Whaley *et al.* (1959a) indicated that most of the cells of maize roots

appeared to have several hundred such structures; other cells certainly have fewer. The manner in which the dictyosomes increase in number is not clear. Whaley *et al.* (1959a) point out that there is no evident reduction of the number of bodies per cell following cell division. However, they and most other workers have failed to observe division, or *de novo* origin, of the dictyosomes. Buvat (1958a) believes that in *Elodea* the dictyosomes divide by constrictions, although his evidence of this process is not convincing.

In contrast with animal cells, in which the Golgi bodies frequently show proximity and orientation with respect to the nucleus or other structures, the dictyosomes of plant cells are usually randomly distributed in the cytoplasm (Buvat, 1958b; Heitz, 1958; Sitte, 1958; Whaley *et al.*, 1959a, 1960). However, in some of the very small unicellular algae studied by Manton and her colleagues (Manton, 1959, 1961; Parke *et al.*, 1959; Manton and Parke, 1960) some restriction in localization of the dictyosomes was noted. This may be a consequence of a necessity for extreme compactness in such cells, rather than from some essential relationship of the position of the dictyosomes and that of other cell organelles. On the other hand, in the somewhat larger cells of *Chlamydomonas*, Sager and Palade (1957) noted that the dictyosomes were regularly in the vicinity of the nucleus. Greenwood (1959) reported that the dictyosomes observed in zoospores of *Vaucheria* were intimately associated with mitochondria. In another example, the dictyosomes of the shoot apex of *Antirrhinum*, a flowering plant, were always observed in the vicinity of the nucleus (Heitz, 1958). However, the same author found no discernible orientation in the many other species of plants which he studied (Heitz, 1958).

In animal cells, the function of the Golgi bodies has long been linked with secretion and excretory activity (cf. Dalton, 1961). However, the role of the dictyosomes in plant cells remains largely unknown. Observations by Mollenhauer *et al.* (1961), noted above, and by Bouck (1962) would be consistent with secretory processes, and Buvat (1958b) has suggested a possible secretory function. The nature of the dictyosomal material observed by Mollenhauer *et al.* (1961) to be deposited at the cell wall has not been determined. The dictyosomes could certainly be associated with deposition of cell wall material, both at cell-plate formation and in differentiating cells. However, it is difficult to believe that this is the case in the maize root cap studied by these workers, inasmuch as the cells of the cap apparently are short lived. It is generally believed that the root cap sloughs off cells as a form of lubrication along the path of the advancing root tip (cf. Esau, 1953;

Meyer and Anderson, 1952). Thus, the vesicles released by dictyosomes of these cells might contain the slippery, mucilagenous substances commonly observed on root caps. Alternatively, the vesicles might release a pectinase which could facilitate separation of the cells from the outer layers of the root cap. As should be clear, however, these

FIG. 14. This section (permanganate-fixed), from a cell of *S. ionantha*, shows an oblique view of a dictyosome (*D*). In addition, several plasmodesmata may be seen at the cell wall (*CW*) in the center of the figure. The endoplasmic reticulum (*ER*) is also well illustrated. Magnification: × 43,000. (Electron micrograph by Ledbetter and Porter.)

suggestions are in the nature of speculation and would represent a rather specialized function of the dictyosomes. But, specialized roles in diverse tissues would not necessarily be incompatible with a general function such as secretion of materials for wall differentiation or cell-plate formation (where there often is a conspicuous concentration of dictyosomes and of the vesicles derived from them). In any case, one cannot avoid

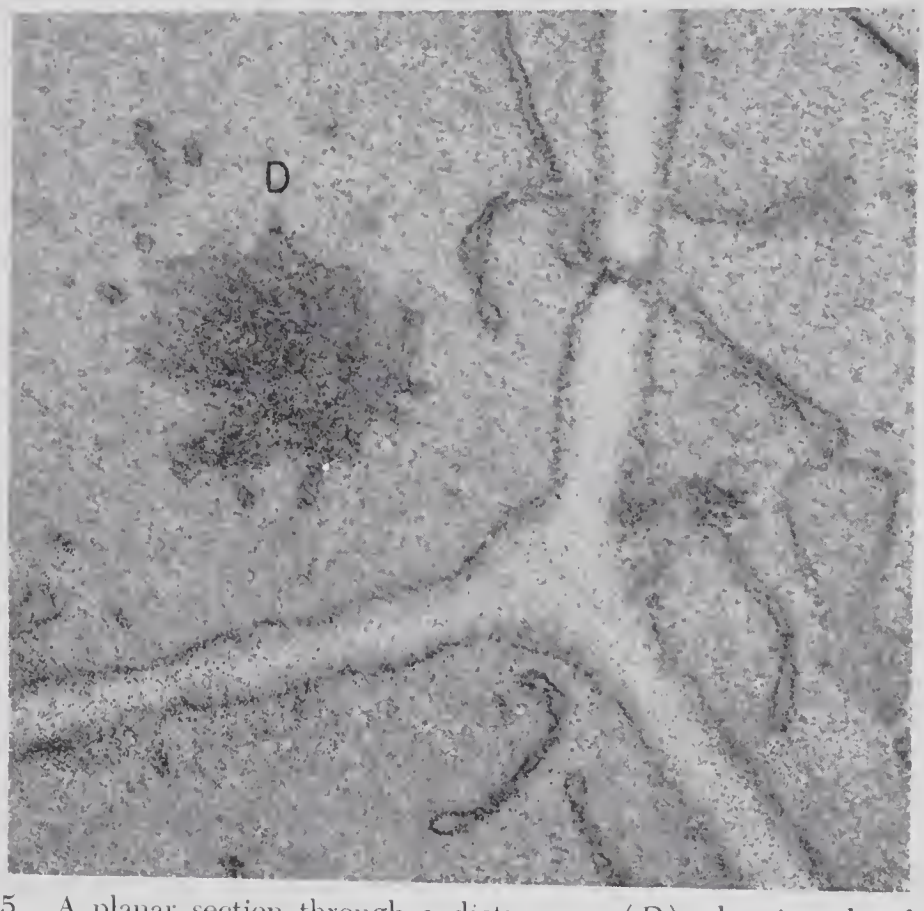

FIG. 15. A planar section through a dictyosome (*D*), showing the disk-shaped appearance in full, surface view. From a permanganate-fixed anther cell, *S. ionantha*. Magnification: × 43,000. (Electron micrograph by Ledbetter and Porter.)

concluding that the role of the dictyosome in the physiology of the plant cell remains almost entirely a matter for future investigation.

C. Vacuoles

1. Distribution and Morphology

First use of the term vacuole is attributed to Dujardin (1841) in referring to the protozoan contractile-vacuole, originally described by Spallanzani in 1776 (cf. Zirkle, 1937). Although contractile vacuoles also have been observed in the zoospores and gametes of diverse algae, as well as in sponges (cf. Jepps, 1947) and in the blood cells of various vertebrates including man (Bessis, 1956), they are not recognized as

FIG. 16. A vacuolate anther cell from *S. ionantha*. The cell contents include amyloplasts (*A*) containing starch granules, in addition to mitochondria (*M*), a nucleus (*N*), two nucleoli (*NL*), etc. The single-membraned tonoplasts (arrows), surrounding the vacuoles, and the single-membraned plasmalemmae are distinctly different from the double membrane surrounding the nucleus. Cells osmium fixed. Magnification: × 8800. (Electron micrograph by Ledbetter and Porter.)

usual components of plant or animal cells in general. The less spectacular plant structure, with which the name vacuole is now customarily associated (Fig. 16), may be observed in the cytoplasm of nearly all plant cells. These somewhat more stationary vacuoles were not described and illustrated until the mid-nineteenth century (Meyen, 1835; Schleiden, 1842), according to Zirkle (1937). Since 1880, however, they have been much studied and the subject, directly and indirectly, of many hundreds of research papers.

In mature cells, where vacuoles are most readily observed, they appear as large, customarily transparent, vesicles surrounded by a membrane. The cells of tissues which contain anthocyanins are often particularly favorable for study inasmuch as the red-purple pigments are localized in the vacuole and sharply set it off from other cellular constituents. The volume of such vacuoles in mature cells is typically greater than that of all other cellular components combined; indeed, it is often nearly equal to the volume of the entire cell. Crafts *et al.* (1949), for example, state that the vacuoles of parenchymatous tissues comprise 70–90% of the cell volume. In such mature cells, the cytoplasm and nucleus occupy only a narrow marginal band pressed against the cell wall.

In meristematic tissues the vacuoles are often quite small (ca. 2–10 μ) and numerous. Their shape is variable, being threadlike in the tips of rose leaflets (Fig. 17) and spherical in many cases such as the root meristem (Fig. 18a). The tiny vacuoles are smallest and most numerous in cells at the extreme apex of the meristem. Here they also appear most deeply pigmented in tissues naturally colored by anthocyanin or cells grown in solutions of vital stains. As the enlarging and differentiating cells become further removed from the tip of the root, the vacuoles increase in size and fuse together (Fig. 18b, c). Simultaneously, the vacuolar pigments become lighter in color, presumably through dilution of their contents. Presently the cells are nearly completely occupied by a few, large, lightly colored vacuoles which are pressed one against another (Fig. 18d, e). In the mature parenchymatous cell, a single, large, pale colored vacuole occupies most of the cell. The remarkable correlation between the increase in volume of the vacuole and that of the cell as a whole during these growth processes will be discussed more fully below.

The ubiquity of the presence of vacuoles throughout the plant kingdom was pointed out by Went (1888). It is now clear that they are found in plants of all groups except the blue-green algae, as noted already. Thus, these organelles are observed in the several other groups of algae, in mosses, liverworts, ferns and fern allies, gymnosperms, and

flowering plants. Furthermore, as reported by Went (1888) and by Hof (1898a, b, c, d), vacuoles are found in all cells within a plant, including the meristematic cells. As noted by Bailey (1930) and Zirkle (1932), many textbooks overlook these early studies and state that vacuoles are found only in mature cells. Nevertheless, the views of Went and Hof have been amply confirmed by recent workers, including

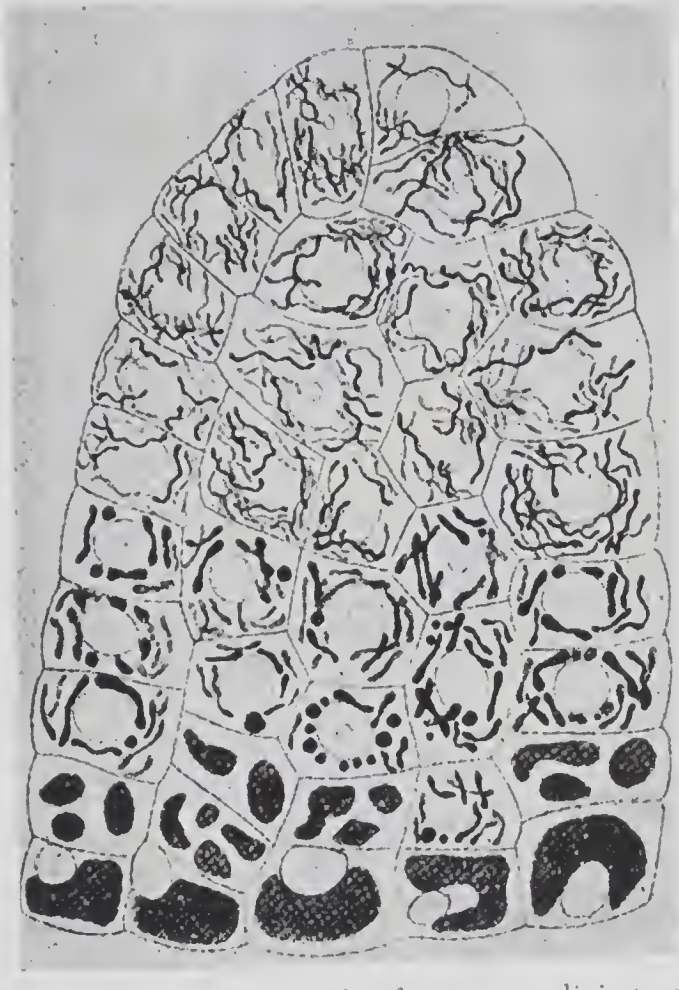

FIG. 17. A section through the tooth of a young, living rose leaflet, showing anthocyanin-containing vacuoles at various stages of development, from filamentous vacuoles to large, single ones. From Guilliermond (1941).

such recognized authorities as Bailey (1930), Guilliermond (1941), Zirkle (1932, 1937), and Küster (1935). Bailey brought attention to the extensive vacuolation of the long slender cambial cells which produce "secondary growth" in woody plants. Guilliermond demonstrated the presence of tiny vacuoles in the red tips of leaf scales from rose plants, in the root meristems of various grasses, and in diverse other meristematic tissues. These studies have also been supported by observation in the electron microscope of meristematic tissues from many groups of plants (Lund *et al.*, 1958; Buvat and Mousseau, 1960; Menke, 1960a; Porter and

Machado, 1960; Whaley *et al.*, 1960; Manton, 1962). In very young cells prepared for examination in the electron microscope, the tiny vacuoles often have a striking stellate appearance, as reported by various workers (e.g., Porter and Machado, 1960; Manton, 1962).

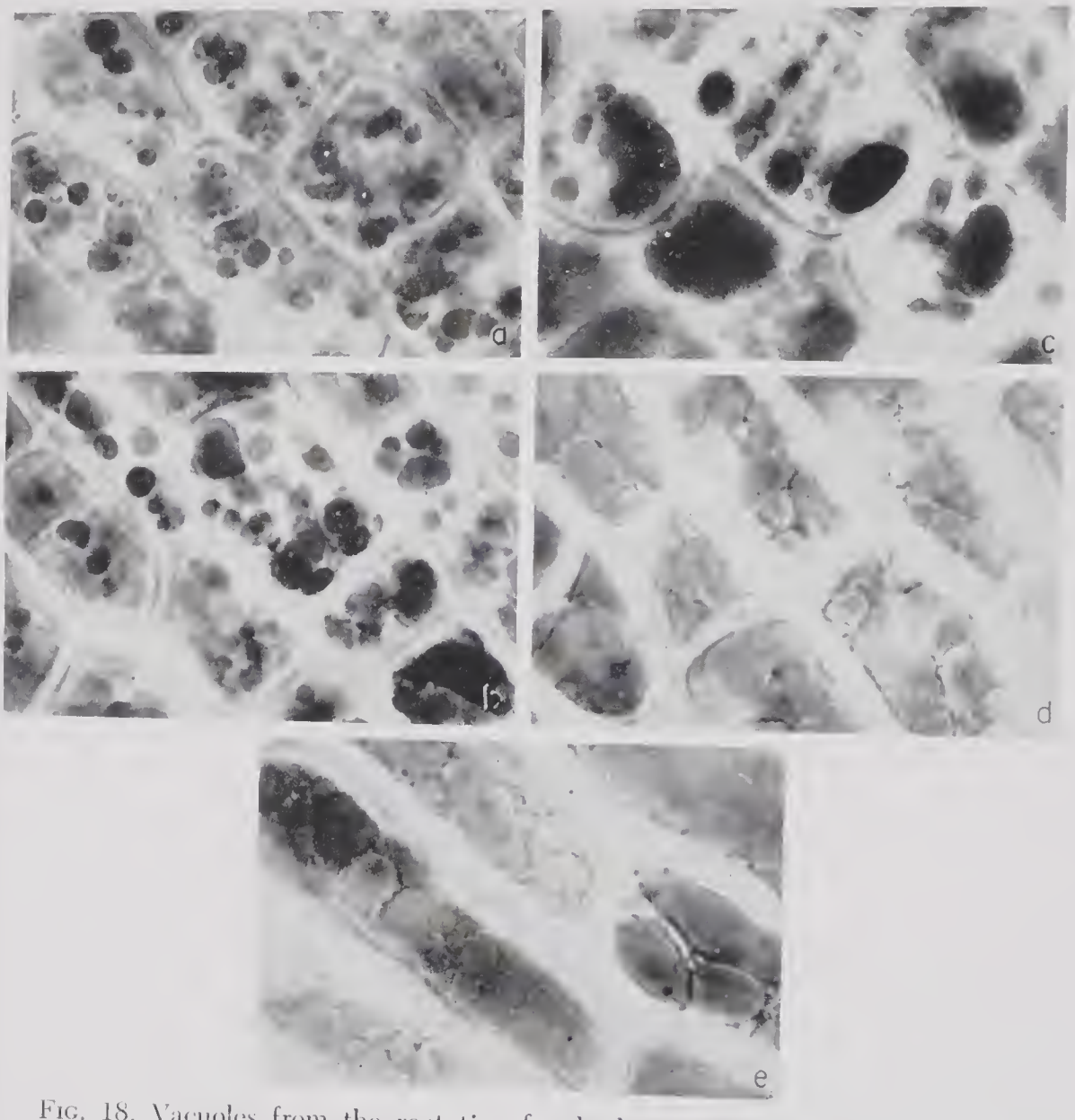

FIG. 18. Vacuoles from the root tip of a barley seedling grown in neutral red solution. The various developmental stages of the vacuole are shown, including very young cells with spherical vacuoles (a), older cells with vacuoles in various stages of growth and fusion (b–d), and cells occupied almost completely by large fusing vacuoles (e).

As a consequence of the shearing forces caused by cytoplasmic streaming (cyclosis), the vacuoles of cells are often quite irregular in form and may be reticulate or drawn out into rod shape (Stocking, 1956a; Zirkle, 1932; Küster, 1935; Bailey, 1930). Indeed, this may be the

basis of the thread-shaped vacuoles of the meristematic cells of *Pinus strobus* and the walnut gland cells shown in Figs. 19 and 20. However, cyclosis is not generally observed in immature cells, and thus the vacuoles are most typically spherical in shape.

Another interesting form of vacuole was originally noted by Darwin in his studies of the tentacles of the insectivorous sundew (*Drosera*) (cf. F. Darwin, 1876). He observed that the anthocyanin-pigmented

FIG. 19. Reticulate vacuoles of meristematic cells from *Pinus strobus*. From Zirkle (1932).

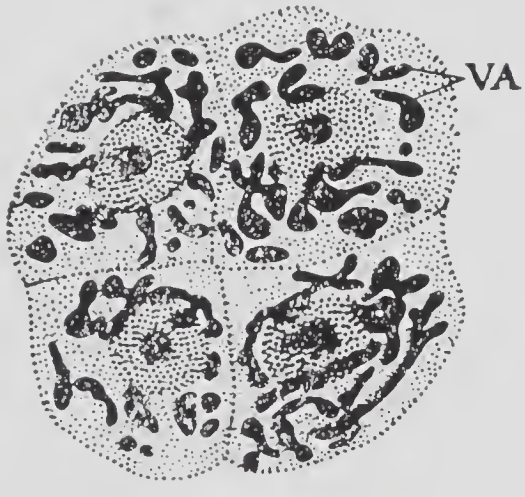

FIG. 20. Filamentous vacuoles (*VA*) from the oil-gland cells of a walnut leaf. From Guilliermond (1941).

contents of cells of the tentacles broke up into numerous granules and filaments when the tentacle was mechanically stimulated. This process has been described by Guilliermond (1941) in the following way: "At the moment of stimulation this vacuole undergoes a great fragmentation. It splits into a large number of small chondriosome-shaped vacuoles. Immediately after stimulation, these minute vacuoles fuse to constitute again a single large vacuole—the cell returns to its initial state." During this process, the cytoplasm takes up water from the vacuole and the vacuole increases in osmotic pressure by about 5 atm. Results of centrifugation studies indicate that the vacuoles of stimulated

tentacles have a greater specific gravity than those of the cytoplasm. The converse is true of the vacuoles of unstimulated tentacles (cf. Guilliermond's discussion, 1941, of the work of Åkerman).

The secretory (or "digestive") cells of the tentacles of *Drosera* and related species have finely divided, granular and filamentous vacuoles

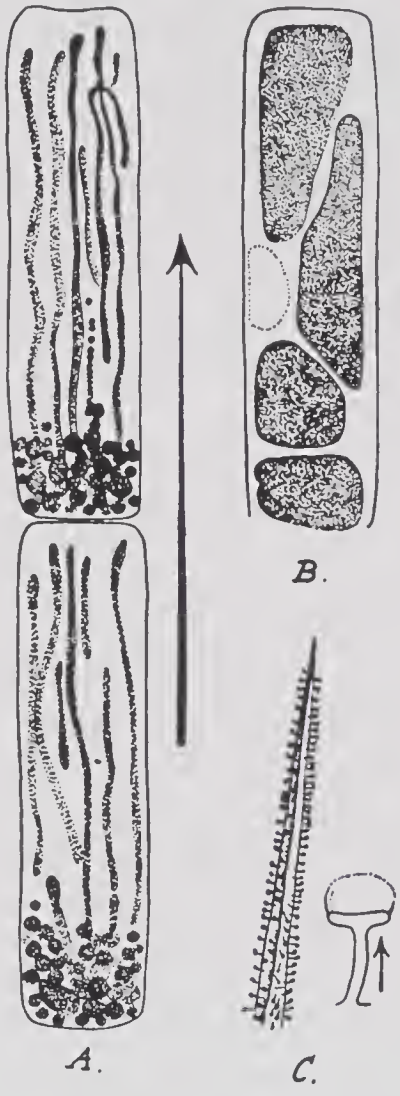

FIG. 21. *Drosophyllum lusitanicum*. Epidermal cells of the pedicel of a tentacle. Vacuoles in gray, arrows indicate the direction of the head of the gland. A, during digestion of a protein; B, during an inactive period; C, tip of a leaf with tentacles; one gland showing pedicel, head, and drop of secretion. From Guilliermond (1941; from Mangenot).

during "digestion" (Fig. 21A). During resting periods the vacuoles are large and fused (Fig. 21B).

2. *The Tonoplast*

The presence of some manner of phase boundary between the vacuole and the cytoplasm has been recognized for more than a century.

It was clearly noted by de Vries (1877a, b, 1884) in his classic studies of plasmolysis. He also discussed the work, from thirty years earlier, of Nägeli and N. Pringsheim (cf. also Hecht, 1912). The latter two workers had investigated the process of plasmolysis, in which water moves out of the vacuole of a cell placed in a hypertonic solution, resulting in decrease in the volume of the vacuole. During this process the protoplast, which had been held against the relatively rigid cell wall by the pressure of the turgid vacuole, shrinks away from the cell wall. This process leaves a readily discernible space between the wall and the cell membrane. The space becomes filled with the plasmolytic solution and the water leaving the vacuole.

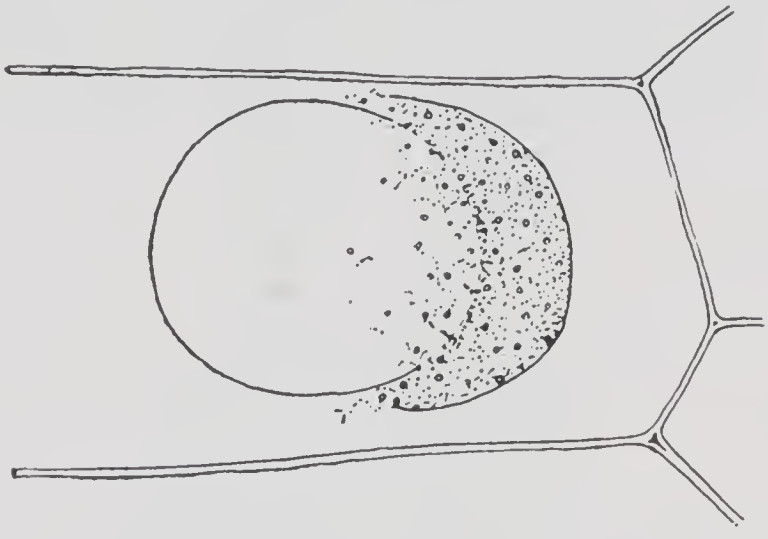

FIG. 22. A plasmolyzed onion cell with its end wall removed and the cytoplasm (stippled) partially teased away from the spherical vacuole by means of a microneedle. From Plowe (1931a).

de Vries (1877a, b, 1884) and Pfeffer (1886, 1900) greatly extended these early observations and recognized that the vacuole acted as an osmometer and possessed a semipermeable membrane. They noted that epidermal cells of such plants as *Tradescantia* and *Begonia* contained red pigments (anthocyanins), which were confined to the vacuole and moved from that structure into the cytoplasm or external medium only upon the death of the cell or upon injury to the tonoplast. Much later, Chambers and Höfler (1931) and Plowe (1931a, b), using techniques similar to those originally employed by Townsend and by Klercker (cf. Townsend, 1897) for isolating whole protoplasts, microsurgically separated the vacuoles of diverse plant cells from the surrounding cytoplasm (Fig. 22). They demonstrated that isolated vacuoles retained their characteristic osmotic properties and also their red anthocyanin pigments for substantial periods of time (see also Eichberger, 1934). Thus, these

experimenters simply and elegantly confirmed the existence of a semipermeable tonoplast.

In addition to the maintenanec of an anthocyanin gradient aeross the tonoplast and to the tonoplast's semipermeability to osmotieally active substances, the outer membrane of the vacuole has a striking response to various dyes. For example, phenol red, cresol red, aniline bluc, and acid fuchsin freely diffuse throughout the interior of the vacuole when mieroinjected there, but do not leave it. Moreover, when sulfonephthalein indicators (which also remained exelusively in the vacuole when injected there) were placed in the cytoplasm, they migrated out of the cytoplasm into the vacuole (Chambers and Chambers, 1961; Chambers and Kcrr, 1932). Thus, there can be little doubt of the physiological existence of a selectively permeable membrane surrounding the vacuole. (Sce also the ion-accumulation properties of the vaeuole, discussed below.)

As seen in the elcctron microscope, the tonoplast appears as a well-dcfined structurc (Fig. 16). On the basis of the initial electron microscope studies, when tcchniques for preparing and sectioning plant materials were less highly developed, several authors reported that the tonoplast was a "double membrane" (Buvat, 1957a; Mercer, 1960; Kaufmann and De, 1956). However, in more recent photographs of adequate magnification, clarity, and resolution, it is evident that the tonoplast is a singlc membrane about 70–100 A. in thiekness as viewed in diverse groups of plants including unicellular and multicellular organisms (Buvat and Mousseau, 1960; Sager and Palade, 1957; Bouek, 1962; Gibbs, 1960; Perner, 1958; Sitte, 1958; Whaley *et al.*, 1960; Manton, 1960; Poux, 1961; and many others).

It is of interest to note that Buvat and his colleagues (Buvat, 1957b; Buvat and Mousseau, 1960) and Poux (1961) maintain that the tonoplast is often eontinuous with the endoplasmie reticulum, as seen in electron micrographs. Thcse authors believe that vacuoles arise through local dilation or expansion of elements of the endoplasmic retieulum. However, thc evidcnce supporting such an origin has not been convincing, and other workers do not concur in the French cytologists' views (e.g., Manton, 1962). Moreover, ribosomes are not assoeiatcd with the tonoplast, whcreas they are generally observcd along the endoplasmie reticulum. It seems likely that the impression of parallel vaeuolar membrancs suggestive of endoplasmic reticulum might result from deformations of the vacuoles during cyclosis (for example, see Poux, 1961, Fig. 7).

The surface tcnsion of the vacuolar membrane has been studied by Pigoń (1950, 1951). He dissected free the vacuoles of plasmolyzed

epidermal cells from onion bulbs (*Allium cepa*) and studied the surface tension of the vacuoles by use of two techniques. One method involved measurement of the curvature of the interphases between the isolated vacuoles, small droplets of oil placed in contact with the vacuoles and the aqueous bathing solution. The other method involved measurement of the radii of curvature of vacuoles pressed between two plates of glass. The first method simultaneously measured elastic tension and surface tension and led to determination of mean values of about 2.5 and 4.1 dynes/cm. for vacuoles in solutions buffered at pH 7.0 and 4.8, respectively. The second method was thought to measure surface tension alone and led to determination of an average value of 0.038 dynes/cm. for 25 vacuoles. However, in each case the range of values was quite large. Furthermore, all measurements appear to have been made upon cells bathed in a medium containing 1 *M* sucrose, which would be strongly plasmolyzing. Plasmolysis and other experimental procedures are known to affect the properties of the tonoplast (Döring, 1932; Stadelmann, 1956). Thus, it is difficult to judge what relation the surface tension of these plasmolyzed vacuoles would have to that in normal, intact cells.

3. *The Contents and Function of the Vacuole*

Both in the algae and in the land plants the composition of the cell sap is highly variable, and Küster (1935) has rightly claimed that the sole substance universally found in vacuoles is water. Members of virtually all groups of biologically synthesized compounds have been reported to be found there. The substances found in vacuoles include dissolved mineral salts, organic acids (chiefly in land plants), amino acids, amides, proteins, peptides, sugars and other carbohydrates, nucleic acids, alkaloids (chiefly in dicotyledonous plants), tannins (especially in land plants, including mosses, ferns, and seed plants), mucilages, glycosides, etc. (cf. Küster, 1935; Guilliermond, 1941; Pisek, 1955). Many of these substances show high taxonomic specificity, whereas others are of widespread distribution.

On the basis of study of both living and histologically prepared cells, it is evident that even a single cell may contain vacuoles with quite different contents (Guilliermond, 1941; Stocking, 1956a; Whaley *et al.*, 1960). In many cases, however, it appears that vacuoles may be divided into two types; one form is rich in tannins and has a low pH, the other a higher pH and is more or less free of tannin (Guilliermond, 1941).

In addition to the substances in solution and in colloidal suspension, vacuoles frequently contain crystalloid substances and concretions of

various sorts (Fig. 23) (cf. Guilliermond, 1941; Küster, 1935; Steffen, 1955). Most commonly seen are proteins, pigments, and salts of organic acids. Anthocyanin crystals are frequently observed, as well as crystals of calcium oxalate, leucine, and asparagine (Guilliermond, 1941).

The aleurone grains, present in all seeds and in the embryo, endosperm, and cotyledons, are very frequently crystalline and have been reported to be dehydrated vacuoles (Dangeard, 1921a, b, c), rich in protein. Upon germination, the aleurone grains are rehydrated and once again become vacuoles (cf. also Guilliermond, 1941; Küster, 1935),

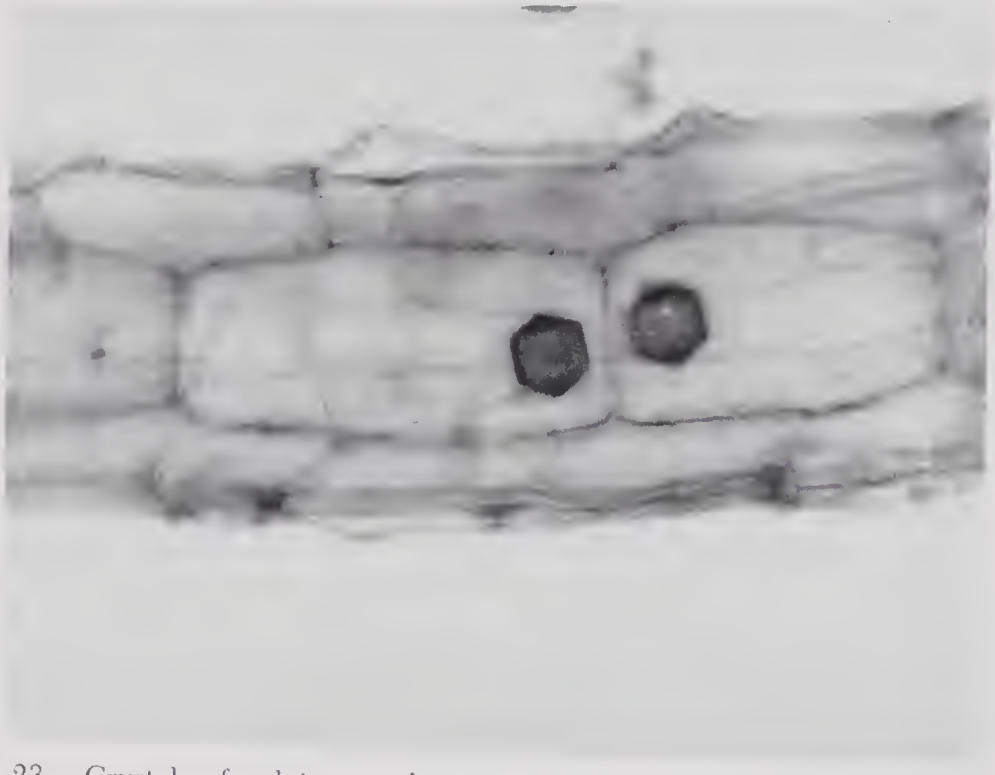

FIG. 23. Crystals of calcium oxalate (?) in the vacuoles of hair cells from the petiole of *Begonia* sp., as seen in polarized light.

although Dangeard's interpretation has been challenged by Steffen and others (cf. Steffen, 1955).

The viscosity of vacuoles varies widely, inasmuch as the vacuoles may be largely composed of water or may become solid through the gelling of colloidal materials (cf. Guilliermond, 1941). The cell sap is, however, most characteristically fluid. By measuring the sedimentation rate of gypsum crystals within the vacuoles of algal cells (Fig. 24), it was possible to make viscosity determinations of the cell sap. Frey-Wyssling (1948) summarizes viscosity values obtained by various workers from the study of algae, mosses, and flowering plants. Viscosities ranged from 1.9 to 2.5 times higher than that of water or of synthetic aqueous solutions of the same concentrations as that of the vacuoles.

Frequently the most striking components of the vacuole are its pigments, chiefly the water-soluble anthocyanins and flavones. These two groups of pigments are structurally closely related. The anthocyanins may be regarded as glycosidic benzopyrylium (flavylium) structures,

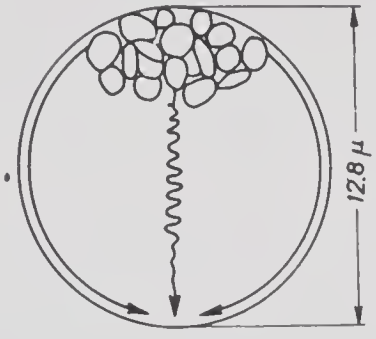

FIG. 24. Sedimentation path of gypsum crystals in the vacuoles of *Closterium*. From Frey-Wyssling (1948).

shown in Fig. 25 in chloride form. The anthocyanins are typically 3,5,7-substituted, commonly having a hydroxyl group at the 7 position, a sugar at the 3 position, and either a hydroxyl group or sugar at the 5 position. The sugar components thus far found are glucose, galactose, rhamnose, and gentiabiose. The anthocyanins further differ from one

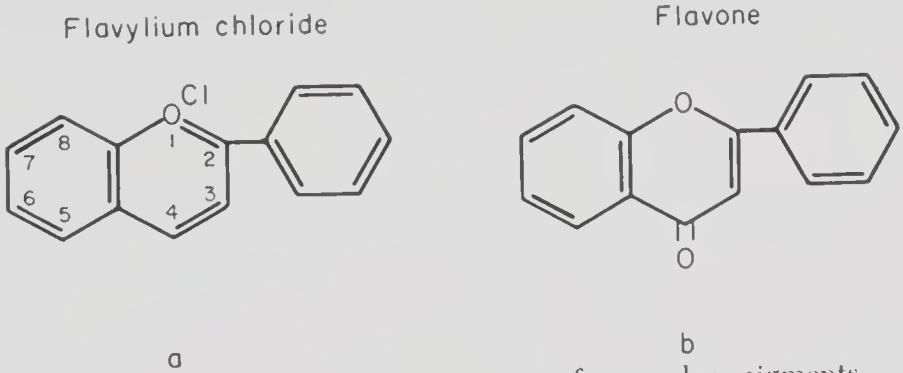

FIG. 25. The chemical structures of vacuolar pigments.

another through the substitution of various combinations of H, OH, or OCH_3 groups at the 3′, 4′, or 5′ positions of the 2-phenyl ring and esterification of the OH groups of the ring nucleus or the sugars with organic acids. Similarly, the yellow flavones are derivatives of 2-phenyl-1,4-benzopyrone (flavone), typically 5,7-dihydroxy substituted. The flavones (Fig. 25) may also be variously substituted with OH or OCH_3 groups at the 3, 3′, and 4 positions and may appear in free form or as glycosides (Blank, 1947; Fruton and Simmonds, 1958).

The diverse shades of red, blue, and violet seen in plants are chiefly due to these pigments, which Tswett (1914) referred to in the title of a paper as "vegetable chameleons," because of their structural variability

and the color changes brought about in them by differences in hydrogen ion concentration and background solution composition.

The pigments are most common in the vacuoles of petals, fruits, and leaves but may occur in any organ of the plant. At least at some developmental stage, virtually all land plants possess cells containing anthocyanins in their vacuoles. This is true of ferns, flowering plants, and gymnosperms. However, scattered examples of anthocyanin-free plants have been reported. Two such groups include members of the cucumber family (Cucurbitaceae) and certain lilies (Amaryllidaceae) (Wheldale, 1916). Furthermore, the anthocyanins appear to be generally absent from the cells of algae and fungi (Blank, 1947), although they may occasionally be present (Smith, 1950). Anthocyanins are very frequently seen in plants growing in deep shade, in alpine and desert areas, and in tropical regions. Histologically, the pigments are most characteristically, but by no means exclusively (e.g., many berries, beet root), localized in the epidermal cell layers, including those of petals, sepals, fruits, leaves, shoots, and roots (Wheldale, 1916; Küster, 1935; Blank, 1947). Detailed discussion of the histological and ecological distribution of anthocyanins can be found in Wheldale's (1916) book.

a. Pigment function. Without doubt, the best-established function of the anthocyanins and flavones is the attraction of pollinators, as first suggested by Sprengel (1793, quoted by Blank, 1947). The bright colors of the floral and reproductive organs of many plants are intimately related with the visual characteristics of the pollinator. For example, bees do not see the color red but have high sensitivity to yellow, blue-green, blue, and various regions of the ultraviolet (von Frisch, 1914, 1960). The vast majority of plants pollinated by bees have flowers colored (i.e., reflecting) yellow, white, blue, and various shades of ultraviolet either as single solid colors or as combinations of them, contrasted against the green foliage background. In this respect, it is of interest that true red flowers are virtually absent from the Northern European flora, which is chiefly bee-pollinated (von Frisch, 1914; Grant, 1950, 1951).

In the tropics and the southern hemisphere, birds rather than bees are the predominant pollinators. Birds are mainly attracted by color rather than by scent; their eyes, in contrast to those of bees, are highly sensitive to red colors. This undoubtedly is the basis for the very high incidence of red floral organs in bird-pollinated plants (von Frisch, 1914). Similarly, "butterfly colors" are red and orange.

As might be expected, the many flowers which are frequented by dusk and night-flying animals such as moths and bats, are colorless.

They are white or shades of white, and many of them are open only during the evening (Grant, 1951). The flowers of plants that are wind-pollinated rarely are colored and, indeed, usually lack petals; examples include the grasses, rushes, sedges, nettles, many hardwood trees, and all conifers (Lovell, 1899; von Frisch, 1914; Grant, 1951).

From the examples cited, it should be apparent that the anthocyanins and flavones, with their diversity of colors and shades within the visual range of the chief pollinators, are of major importance to the plant. This relationship between animal color-perception and plant pigmentation will certainly become increasingly clear as the perceptual abilities of various groups of animals become more fully elucidated.

By use of light filters consisting of suitable solutions of dyes, Pringsheim (1881, 1882) obtained results which he interpreted as indicating that chlorophyll was decolorized and cells killed in bright yellow, green, or blue light. He maintained that red light alone was harmless to cells. On the basis of his interpretation of these results, which were obtained without equalization of the energy at the various wavelengths, he postulated that the red-purple anthocyanins act as a screen to protect the cells from harmful yellow, green, and blue radiation. Such an interpretation would be consistent with the predominantly epidermal localization of the pigments. However, it is now clear that red light, rather than having the least affect at high intensity, is actually the color chiefly causing chlorophyll to bleach. Despite Pringsheim's misinterpretation, the concept of the role of the vacuolar pigments as *some* manner of protective light filter, in the physiology of the plant, has persisted.

It frequently has been suggested that anthocyanins and flavones protect various growth processes, including even the action of specific enzymes, from the harmful effects of ultraviolet radiation (Popp and Brown, 1936). Pick (1883a, b, c) suggested that the hydrolysis of starch in the cell was increased by red light. Koning and Heinsius (1903) noted that the diastase content of leaves was always higher in red leaves than in green ones and that the enzyme was inactivated by ultraviolet radiation (see also Shomer, 1936). The inhibition could be prevented by passage of the light through filters containing anthocyanin solutions. Anthocyanins and flavones strongly absorb in the ultraviolet, the anthocyanins having absorption maxima around 270 mμ in addition to those between 500 and 520 mμ (Sehou, 1927). Furthermore, Metzner (1930) has demonstrated that the cell saps of epidermal cells of many plants are essentially impenetrable to ultraviolet wavelengths between 3500 and 4000 A. However, there is no satisfactory evidence that these par-

ticular wavelengths are inhibitory to any physiological processes (Popp and Brown, 1936). It is interesting to note in passing that many high-alpine plants, for example, the Sierra-Nevada monkey flower, *Mimulus whitneyi*, has red foliage at elevations of 7000–12,000 feet, where ultraviolet intensity is high. When these plants are grown from seed at sea level, they are nearly free of red pigmentation. Similar observations upon European alpine plants are well known.

Went (1961) demonstrated that the growth of diverse plants was markedly inhibited by bright green light and claimed that "one may expect to find this inhibition in nature, where the intensity of the green portions of the sun's rays is high." He suggested that the anthocyanins protect the cell from the unexplained inhibitory effect of green wavelengths.

Stahl (1896; cf. Wheldale, 1916, p. 134) found significantly higher temperatures in the red leaves of plants than in comparable green leaves. Similarly, Smith (1909) found a clear relationship between the temperature of leaves of diverse species (cf. also Blank, 1947). On the basis of these and similar studies, it has been suggested that anthocyanins cause an increase in temperature in alpine vegetation and in the red leaf buds of many temperate plants in the spring. The higher temperature is supposed favorably to increase the metabolic rate of these plants. Another suggestion is that the increased temperature enhances the vapor pressure gradient from the inside to the outside of the leaf and thus is of substantial importance in transpiration (Blank, 1947; Kosaka, 1933). At an outside temperature of 20°, a few degrees difference in the temperature of the leaf would result in a considerable vapor pressure gradient between the inside of the leaf and the surrounding atmosphere (cf. Curtis, 1936). Such a moisture gradient would be of considerable importance in moist environments where relative humidity is generally near saturation. It will be recalled that such ecological niches are rich in red-colored plants.

In summary, it must be said that the functions of the anthocyanins are probably manifold, but exact, reliable, experimental data demonstrating specific roles are only infrequently provided. The best-documented role of these pigments is that of pollinator attraction. The many suggested physiological and biochemical roles, while imaginative and interesting, cannot be regarded as well established.

4. Characterization of Contents

In the main, knowledge of the dissolved contents of the vacuole stems from the research of physiologists interested in the osmotic and

electrical properties of the cell and from interest in the relationship of the presence of osmotically active substances to habitats occupied by plants. The presence of osmotically active substances is of fundamental significance in the very important process of cell elongation, in tolerance by plants of saline growth conditions, in resistance of the plant to desiccation, indirectly in spindle orientation in unequal cell divisions leading to cellular differentiation, in regulation of transpiration through opening and closing of stomata, and in maintenance of cell turgor, and thus in all the mechanical functions of the *whole plant*.

Three techniques have been widely employed in the determination of the properties of the dissolved contents of the vacuole. The first, which has been critically reviewed and discussed by Blum (1958) and is known as the *incipient plasmolysis* method, is used only to determine osmotic pressure. It consists in observing tissues placed in a graded concentration series of a plasmolyte such as sucrose. In general, mineral salts are avoided because they penetrate the cell membranes too quickly (Blum, 1958). After a suitable equilibration period, usually about 30 minutes (Bennet-Clark *et al.*, 1936; Currier, 1944; Stocking, 1956b; but see Blum, 1958, p. 25), the tissues are examined microscopically to determine at which sugar concentration 50% of the cells first show evidence of plasmolysis. That sugar concentration is regarded as isotonic with the vacuolar contents. Suitable correction factors have been employed for errors resulting from decrease in cell volume (often 10–30%) when turgor pressure decreases and the distended cell wall shrinks (Blum, 1958; Currier, 1944; Stocking, 1956b). The method has the particular advantages of being utilizable on undamaged living cells and of being applicable to the study of individual cells (e.g., the guard cells of leaves). A disadvantage results in some species and tissues from the tendency of the cytoplasm to adhere to the cell wall. This tendency, as well as partial penetration of plasmolytes other than sucrose, may in part underlie observations that the plasmolytic method leads to somewhat higher osmotic pressure values than the cryoscopic methods described below (Bennet-Clark *et al.*, 1936; Crafts *et al.*, 1949; Currier, 1944; Stocking, 1956b).

The two other methods of measuring the osmotic pressure and chemically characterizing the contents of the vacuole involve removing the cell sap from the vacuole, on the one hand by means of micropipettes or by carefully opening individual cells, on the other hand by subjecting whole tissues to sufficient pressure to cause cell rupture with release of the cell sap. Once the sap is obtained, its osmotic pressure may be determined cryoscopically (Dixon, 1914; Dixon and Atkins, 1910; Gortner

and Harris, 1914). These two methods have the important advantage of permitting quantitative determination of the chemical composition of the vacuolar fluid.

Because of the simplicity of the pressure method, it has been extensively used. However, various workers have been justifiably critical of the interpretation of data obtained by this method. Dixon (1914) pressed sap from beet roots and also from the leaves of *Chamaerops* and found that the extracted saps did not bring about plasmolysis of the tissues from which they had been taken. Bennet-Clark *et al.* (1936) confirmed Dixon's observations on beet root. Cryoscopically, Bennet-Clark and his co-workers found an osmotic pressure of 9.5 atm. for expressed sap and 12.6 atm. for sap measured by the incipient plasmolysis technique. Although plasmolytic values were higher than cryoscopic values for the roots of beet and swede and for the petiole of *Begonia*, the values were the same by each method for tissues from *Caladium* and rhubarb (*Rheum*) and the sap pressed from these latter two plants brought about 50% plasmolysis of similar tissues from the same species of plants.

Currier (1944) made a careful study of the factors causing differences in results as measured in the two different methods. He concluded that in none of the cases he studied could the differences entirely be explained by changes in cell volume and protoplast-cell wall adhesion during plasmolysis. Rather, the basis was largely in contamination of the expressed sap by protoplasmic liquid. Indeed, Dixon (1914) long ago pointed out that the composition of expressed sap is dependent upon the pressure applied to tissues and the number of times they are pressed. In initial pressings, almost pure water was obtained by him, presumably from vascular cells and from intercellular spaces. Subsequent pressings included the contents of increasing numbers of ruptured cells. On the basis of these and numerous other studies, it is generally agreed that the press method is less reliable than the plasmolytic method (cf., also, Crafts *et al.*, 1949).

Somewhat more precise information may be obtained by the relatively difficult and laborious technique of removal of the vacuolar fluid from individual cells. This has been done by insertion of micropipettes, in study of the intracellular localization of tobacco-mosaic virus (Livingston and Duggar, 1934) and by carefully breaking the very large cells of algae such as *Nitella*, *Valonia*, and *Chara* (Hoagland and Davis, 1923; Osterhout, 1922; Collander, 1936; Blinks and Nielsen, 1940). The size of these algal cells made feasible such careful study. However, the vacuoles

of most plants are far too small to allow the technique to be commonly employed.

By means of the various techniques cited above, it has been established that the cell saps of the vast majority of nonaquatic plants fall within the range 5–30 atm. and perhaps most typically are around 10 atm. (by comparison, the osmotic pressure of human blood is ca. 7.5 atm.). Plants growing under very saline or under desert conditions sometimes have higher values. For example, the East African mangrove, *Avicennia marina*, may reach values of 62 atm. (Walter and Steiner, 1936), and Harris (1934) recorded values of 202.5 atm. in the desert plant *Atriplex*. Zeuch (1934) recorded a value of more than 300 atm. in living cells of the airborne alga *Pleurococcus vulgaris*. However, such high values are curiosities rather than typical values.

The detailed analysis of the sap composition underlying the osmotic pressure of the cell has been a continuing interest of physiologists for nearly a century. The foundation for this analysis was laid in a series of penetrating studies by the versatile Dutch botanist Hugo de Vries (1877a, b, 1884). de Vries pressed sap from the leaves and stems of many different plants. He then determined the relative ability of these cell saps to plasmolyze epidermal cells of *Rhoeo*, in comparison with the effect of various concentrations of aqueous solutions of KNO_3 upon *Rhoeo* cells. He quantitatively analyzed the cell sap for its chief constituents. Then he determined the relative ability of these individual components to plasmolyze *Rhoeo* cells. Thus, he could calculate the portion of the total osmotic pressure contributed by the various sap solutes. By this technique he demonstrated that the chief osmotically active solutes in the vacuole were organic acids (mainly malic), inorganic salts (mainly of Ca, Mg, and K), and sugars.

Since the days of de Vries's studies, many plants have been investigated. From the results of these investigations it is clear that the osmotic pressure and the composition of the vacuole are greatly affected by many environmental conditions (cf. Blum, 1958; Pisek, 1955; Stocking, 1956b). Nonetheless, de Vries's observation of the importance of inorganic ions, sugars, and organic acids in the osmotic properties of the plant cell has been strongly supported. Table III summarizes data from several workers. It can be seen that the sap components listed by de Vries account for two-thirds, or more, of the osmotic values reported. Knodel's data, which include tests for a wide range of substances and which are more complete than the other data shown, account for at least 79% of the osmotic values of the cell sap of six different plant species. It can be seen that the two lowest values, for

TABLE III

THE OSMOTICALLY ACTIVE COMPONENTS OF THE CELL SAPS OF HIGHER PLANTS

A. (After Pittius, 1934)
(data in atm.)

Plant	Date	Sugars	Organic acids	Inorganic ions	Total	Cryoscopic value	%[c]
Hedera helix (ivy)	Feb.	6.4	6.0	3.3	15.7	18.8	62
H. helix	July	1.9	5.4	4.0	11.3	12.2	93
Ilex aquifolium (holly)	Feb.	6.5	5.3	3.0	14.8	21.6	68
I. aquifolium	July	1.9	4.2	3.3	9.4	12.8	73

B. (After Pisek, 1950)
(approximate values in atm.)

Plant	Sugars	Acids and salts	Total	Cryoscopic value	%[c]
Rhododendron ferrugineum	8.5	8.5	17	20	85
Pinus cembra	9	5	14	17.5	80

C. (After Knodel, 1938)
(data in atm.)

Plant	Sugars	Organic acids	Salts	Total	Cryoscopic value	%[c]
Elder	3.90	1.80	8.42[a, b]	14.1	17.8	79
Maize	3.10	1.34	3.72[a, b]	8.2	10.4	79
Turnip	1.98	0.33	7.11[a]	9.4	10.2	92
Rye	4.31	2.24	6.71[b]	13.3	15.9	88
Fir	9.95	2.65	4.66	17.3	18.6	93
Box	8.58	1.34	9.85	19.8	22.7	87

[a] Cl− not determined.

[b] Ca not determined.

[c] % = The percent of the total osmotic pressure (cryoscopic value) contributed by sugars, acids, and salts.

maize and elder, do not include determination for calcium or chloride ion, which amounted to ca. 1.5 atm. and 2 atm., respectively, in those plants in which determinations were made. Thus, if such values are added to the total for these two plants, the percentages for all plants are quite high. This would almost certainly be true for the data of Pisek and of Pittius (1934), as well. Pisek (1950) also found that the three major groups of components accounted for 80–100% of the pressure of *Rhododendron* at various seasons.

TABLE IV
THE OSMOTIC PRESSURE OF SUCROSE SOLUTIONS AT 20°[a]

Mol.	Osmotic pressure (atm.)	Mol.	Osmotic pressure (atm.)
0.00	0.0	0.80	25.9
0.10	2.7	0.90	30.4
0.20	5.8	1.00	35.2
0.30	8.2	1.50	66.6
0.40	11.2	2.00	117.5
0.50	14.5	2.50	203.1
0.60	18.0		
0.70	21.8		

[a] After Blum (1958).

Osterhout (1922) studied the composition of the vacuolar contents of the marine alga *Valonia*, collected at Bermuda. Each cell yielded several milliliters of sap, analysis of which led to the data shown in Table V, part A. As can be seen, potassium was many times higher in concentration in the vacuole than in sea water, whereas sodium, calcium, and sulfate were lower.

Hoagland and Davis (1923) studied the contents of the large cells 1–3 inches long, of the freshwater pond alga *Nitella clavata* and found an osmotic pressure twenty-five times that of the pond water. Comparison of the composition of the pond water and the cell sap is shown in Table V, part B. On the basis of these data, Hoagland and Davis synthetically composed an artificial "sap" which was found to have nearly the same conductivity and osmotic properties as true cell sap. Similar results have been obtained in comparison of natural and synthetic saps from higher plant vacuoles as well (Pisek, 1950; Pittius, 1934). Thus, it appears that the ions in vacuoles are in free solution. These workers also note that barley and wheat, like *Nitella*, show a preferential accumulation of potassium, sodium, and chloride. Land plants in general, however, balance their potassium with organic acids, whereas the algae do so with chloride.

Yet a third large-celled alga, *Hydrodictyon patenaeforme*, had the sap composition shown in Table V, part C. Blinks and Nielsen (1940) stated that both electrical conductivity measurements and osmotic pressure measurements of the cell sap indicated that the inorganic ions

TABLE V

THE IONIC CONTENTS OF THE VACUOLES OF DIVERSE ALGAE

A. (After Osterhout, 1922)
(molecular proportions, expressed as % of Cl)

Ion	Cell sap	Sea water	Ratio cell sap:sea water
Cl	100	100	1.0
Na	15.08	85.87	—
K	86.24	2.15	—
Ca	0.288	2.05	—
SO_4	0.0087	6.26	—

B. (After Hoagland and Davis, 1923)
(in milliequivalents)

Ion	Cell sap	Pond water	Ratio cell sap:pond water
Cl	9.7	0.9	11
Na	10.0	0.2	50
K	54.3	—[a]	—
Ca	20.5	1.6	13
Mg	35.4	3.4	10
SO_4	16.7	0.7	24
PO_4	3.7	0.004	925
NO_3	0	0.5	—

C. (After Blinks and Nielsen, 1940)
(moles per liter)

Ion	Cell sap	Pond water	Ratio cell sap:pond water
Cl	0.063	0.00108	81
K	0.101	0.000019	5300

[a] No detectable K.

were in free solution. On occasion, the sap was as much as 4000 times as rich in potassium as the pond water.

In a detailed investigation of cells from various saltwater and freshwater species of the Characeae, Collander (1936) reported the data shown in Table VI. Again it is evident that sodium, potassium, and chloride ions are particularly abundant in algal cell sap. Comparison of the saltwater and freshwater algae indicated that sap composition

TABLE VI

THE IONIC COMPONENTS OF ALGAL CELL SAP[a, b]

Species	Cl			Na			K			Mg			Ca		
	CS[c]	W[c]	R[c]	CS	W	R	CS	W	R	CS	W	R	CS	W	R
Fresh water															
Chara ceratophylla	176	0.13	1350	84	0.21	400	77	0.04	1900	18	2.0	9	13	3.3	4
C. coronata	141	—	—	44	—	—	86	—	—	—	—	—	18	—	—
Tolypellopsis stelligera	127	0.11	1150	19	0.19	100	128	0.04	3200	21	0.5	42	16	1.1	15
Nitella flexilis	131	0.04	3300	63	0.16	390	58	0.03	1900	—	0.2	—	17	0.2	77
N. gracilis	194	0.12	1600	8	0.26	31	128	0.1	13000	—	0.2	—	32	0.65	49
N. clavata	91	0.9	100	10	0.2	50	54	[d]	—	35	3.4	10	21	1.6	13
Brackish water															
C. ceratophylla	232	80	2.9	148	68	2.2	69	1.4	49.3	26	14.0	1.9	11	3.8	2.9
C. baltica	236	80	3.0	144	68	2.2	58	1.4	41.4	—	14.0	—	29	3.8	7.6
Tolypella nidifica	235	80	3.0	55	68	0.8	107	1.4	76.4	—	14.0	—	11	3.8	2.9
N. hyalina	184	50	3.7	97	43	2.3	52	0.9	57.8	—	9	—	8	2.4	3.3
C. ceratophylla	208	36	5.8	126	31	4.1	61	0.6	101.6	20	6.5	3.1	11	2.0	5.5
Tolypellopsis stelligera	203	36	5.6	41	31	1.3	140	0.6	230	9	6.5	1.4	12	2.0	6.0

[a] After Collander (1936).

[b] Data in milliequivalents.

[c] CS = Cell sap; W = sea water or fresh water, as appropriate; R = ratio of cell sap to water.

[d] Not detectable.

and concentration in these two groups were similar, whereas external solution concentration was very much lower for the freshwater forms. Thus, the ratio of cell sap concentration to that of the bathing solution was much higher in freshwater plants than in saltwater plants.

Although the presence of some iodine has been reported in studies of the various Characeae, it often is present at very high levels in the cells of large species of the brown algae (Phaeophyceae). However, the form in which the iodine occurs has not been clearly established, according to Fritsch (1959).

In a study of cell saps pressed from more than 200 species of herbaceous plants including mosses, ferns, and flowering plants, Iljin (1932) found that mineral salts (K, Ca, Na) generally contributed the most important portion of the osmotic activity. In 84% of the species, the equivalent molar concentration of salts was 1–40 times greater than that of sugars. In most of these species, as has already been shown for selected algae, potassium ion was present at very high concentration; this has also been found to be the case in other land plants (Collander, 1941; Iljin, 1932). Substantial quantities of calcium, magnesium, and nitrate also are commonly found in the sap of land plants, but the presence of sulfate and phosphate is more variable (Pisek, 1955; Hoagland and Davis, 1923).

In part, the quantity of various ions in the cell sap is related to the composition of the solution or soil in which the plant is grown (Collander, 1941; Hoagland and Davis, 1923). Similarly, the osmotic pressure of the sap is closely related to that of the solution (Blum, 1958; Eaton, 1943; Stocking, 1956b). On the other hand, when plants are grown in identical synthetic nutrient environments, there are often clear species differences in the accumulation of various elements. For example, members of the Chenopodiaceae have an unusually high level of magnesium compared with other species of plants grown under similar conditions (Collander, 1941). Also, the wild legume *Astragalus bisulcatus*, which has been found to contain as much as 8400 p.p.m. of selenium, was 500 times richer in selenium than the soil (2.1 p.p.m.) in which it was growing; whereas the species *A. missouriensis* completely failed to accumulate selenium when grown under comparable conditions (Trelease and Martin, 1936). Similarly, plants native to brackish marshes or to dry, salty soils (halophytes) accumulate far more sodium and chloride ions than other plants, even when both groups are grown under identical conditions (Collander, 1941; Steiner, 1939). Indeed, Collander (1941) found that halophytes contained more sodium even when several other alkali cations were equally available. As often quoted, Walter and

Steiner (1936) found that in such halophytes as the East African mangrove the osmotic pressure due to sodium ion alone could be calculated to be as high as 30 atm. These trees grow in sea water (osmotic pressure 22.4–22.9 atm.) and in general have osmotic pressure of 30–35 atm., of which about two-thirds is contributed by chloride ions. However, one mangrove, *Avicennia marina*, actually attained measured osmotic pressure values as high as 62 atm., as pointed out above.

Many varied sugars have been detected in the cell sap of different groups of plants. Chief among these sugars are sucrose, glucose, and fructose, which appear to be nearly ubiquitous in land plants. In the algae they are less common. Substantial quantities of mannitol are often found in the brown algae and floridoside, a galactoside of glycerol, is present in many red algae (Fritsch, 1959; Smith, 1950).

Iljin's (1932) data, from more than 200 species of herbaceous plants, indicated that sugars are considerably less important than salts in the osmotic properties of the cells of herbaecous plants. However, sugars were present in these plants at significant levels. In contrast with herbaceous plants, trees, shrubs, and evergreen and xerophytic plants seem to accumulate substantial concentrations of sugars (Steiner, 1933; Pittius, 1934; Ulmer, 1937; Pisek, 1950, 1955; Iljin, 1957). Examples are shown in Fig. 26 and Tables III and VII. It is evident in most of the evergreen plants listed that sugars comprise a very significant part of the osmotically active sap components. Pisek (1950, 1955) reports that in various members of the *Rhododendron* family (Ericaeeae) sugar concentration rarely falls below 0.3 M (= 8.2 atm.) and increases to as much as 0.7 M (= 2.18 atm.), which represents 70–75% of the osmotic pressure of the cell sap.

The seasonal variation in sugar content, related in evergreens to temperature, is considerable and has been extensively studied (Blum, 1916, 1958; Pisek, 1950, 1955; Steiner, 1933, 1939; Levitt, 1951; Iljin, 1957; Walter, 1929a, b). The high winter values and low summer values in Table III, Fig. 26, and especially in Table VII characterize the vacuolar saps from many plants. By way of contrast, Pisek (1950) found that the other osmotically active sap components showed comparatively little seasonal variation, although Tsuda (1961) found about a twofold winter increase in NaCl content in diverse halophytes.

As with sugars, many different organic acids are found in both sea and land plant cells. The most prevalent acids are the dicarboxylic acids, oxalic, malic, and tartaric, and the tricarboxylic acid, citric acid (Pisek, 1955; Vickery and Pucher, 1940). Of these, malic acid, as pointed out by de Vries (1884), is the dominant acid of higher plants, although one

of the other three acids is occasionally present in at least as great amount (Pierce and Appelman, 1943; Pisek, 1950; Stafford, 1959). Tartrate is present in the thallophytes (Pisek, 1955) and, in flowering plants, is probably found in large quantities in only the grape, geranium, and

TABLE VII
THE SUGAR COMPONENT OF THE OSMOTIC PRESSURE OF PLANTS IN SUMMER AND WINTER[a]

Plant	Osmotic pressure (atm.)	Sugars	Per cent	Season[d]
Petunia sp.[b]	11.0	2.6	23.6	W
	8.0	0.5	6.3	S
Verbena sp.[b]	11.7	2.0	17.9	W
	10.4	0.4	3.8	S
Vinca minor[b]	14.7	2.2	14.9	W
	11.7	0.2	1.7	S
Phyllostachys aurea[b]	21.9	4.3	19.6	W
	16.6	1.1	6.6	S
Aucuba japonica[b]	19.2	6.4	33.4	W
	11.2	0.3	1.8	S
Magnolia grandiflora[c]	15.8	3.3	20.9	W
	11.2	0.7	6.4	S
Laurus nobilis[c]	18.9	6.3	33.3	W
	14.1	2.8	19.8	S
Prunus laurocerasus[c]	17.1	7.3	42.6	W
	13.3	1.7	12.8	S
Prunus lusitanica[c]	17.8	5.0	28.1	W
	12.8	1.0	7.8	S
Ilex aquifolium[c]	20.4	13.3	65.2	W
	13.6	4.7	34.6	S
Taxus baccata[c]	27.0	11.0	40.7	W
	21.0	4.0	19.0	S

[a] After Steiner (1933).
[b] Herbaceous plants.
[c] Evergreen plants or trees.
[d] W = winter; S = summer.

pea families (Vitaceae, Geraniaceae, and Leguminosae) according to Stafford (1959).

The hydrogen ion concentration of the cell sap has been the subject of an immense number of studies. Hurd-Karrer (1939) cites from the literature more than 200 determinations and Drawert (1955) well over 100.

Many of the early observations (e.g., Haas, 1916) took advantage of

the presence in the sap of the naturally occurring "pH indicators," the anthocyanins. However, the technique of observing the color of the sap is beset by many difficulties. The color of the anthocyanins in the vacuole is not only dependent upon the pH of the vacuolar sap but is

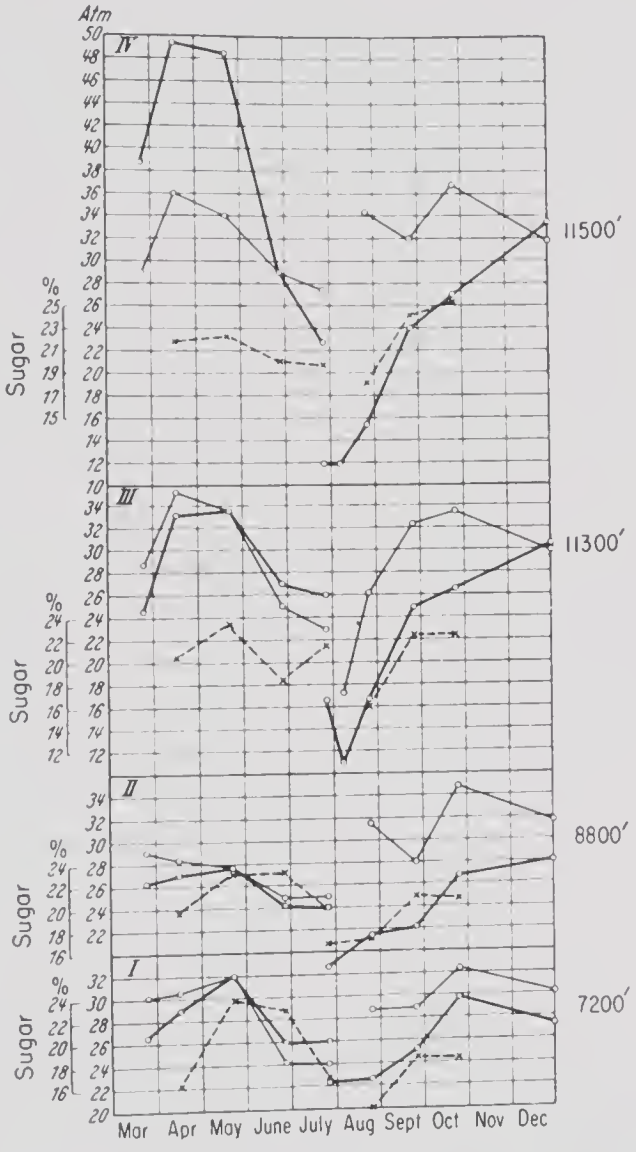

FIG. 26. Osmotic pressure and sugar and water content in the needles of *Picea engelmanni* collected from various elevations (right) during the course of a year. KEY: ——○—— = osmotic pressure (atm.); ——○—— = osmotic pressure compensated for variation in cell water content (atm.); - - - - × - - - - = sugar content of the cell sap, in per cent (left). From Steiner (1933).

affected by the presence and concentration of various copigments such as tannins, flavenols, and by the presence of metal ions, such as aluminum and iron (cf. Beale *et al.*, 1939; Blank, 1947; Drawert, 1955).

More satisfactory data was obtained by Plowe (1931b), Chambers and Kerr (1932), and others by injecting "vital dyes" into the vacuoles

of plants by means of micropipettes (cf. Chambers and Chambers, 1961). The vacuoles of root hairs of *Limnobium*, for example, were found to be pH 5.2 ± 0.2. However, this technique is difficult and time consuming, and thus has not been widely employed. By far, the majority of pH determinations have been made upon sap *pressed* from plant tissues. Values obtained by this method are subject to many criticisms similar to those discussed above for osmotic studies of expressed sap. However, as determined by this method, the great majority of land plants have pH values between 5.0 and 6.5 (Drawert, 1955; Hurd-Karrer, 1939), although scattered species possess higher or lower values. The lowest values are found in species of *Begonia* (ca. pH 1.0), *Oxalis* (ca. pH 2.0), and *Citrus*, the common lime (pH 2.0–2.5). Very few alkaline values have been reported. In the algae, by contrast, the sap pH is generally neutral or even slightly alkaline (Fritsch, 1959), although *Desmarestia*, a brown alga, was reported to be about pH 1.0 (Wirth and Rigg, 1937) and *Nitella* around pH 5.6 (Irwin, 1923).

The function of the organic acids, aside from their contribution to the osmotic pressure of the cell, is largely uncertain, and the acids are most likely *excreted* into the vacuole. It has occasionally been suggested that the acid cell sap might act as an "immunological" factor in parasite attack or as a deterrent to animal foragers. However, there is at present little experimental support for these suggestions.

In some cases (cf. Blank, 1947; Small, 1955) flower color has been observed to change during the course of a day, as a consequence of changes in sap pH. Freshly opened flowers of *Ipomoea leari* are pink (pH 6.0); during the day the color changes to blue and the pH may rise as high as 7.8 (Smith, 1931). Whether or not these changes are of any significance in such processes as pollinator attraction is unknown. It seems much more probable that diurnal variations in pH are simply a consequence of the daily changes in metabolism accompanying photosynthesis.

Differences in the pH of various tissues of a plant at diverse developmental stages have been studied both in flowering plants such as the sunflower and in the thalli of algae such as Laminariales (Martin, 1927; Small, 1955). However, it is unclear whether these variations in hydrogen ion concentration are in themselves of physiological importance or by-products of metabolic processes.

Frost and drought resistance are generally paralleled by high cell sap osmotic pressure, chiefly related to increase in the concentration of sugars (Iljin, 1957; Levitt, 1956; Pisek, 1955). Contributing to high sugar concentration, on a dry weight basis, are starch hydrolysis at low

temperature (Iljin, 1957; Levitt, 1956), a high ratio of photosynthesis to respiration, at low temperature (Levitt, 1956), and decrease in water availability (Iljin, 1957; Kramer, 1949). Although resistance to cold and drought is closely related to high osmotic pressure, the nature of the relationship is not clear. It has been commonly believed that the osmotic contents of the cell sap lower its freezing point and prevent bursting of the cell. However, this is not at all probable. Relatively few plants have osmotic pressures above 36 atm., and it can be readily calculated from the familiar freezing-point depression formula that the osmotic pressure of an aqueous solution is equal to twelve times the freezing-point depression (at 0°C.). Thus, cell sap with an osmotic pressure of 36 atm. would freeze at 3° below 0° C. This cannot be regarded as a significant contribution to resistance to freezing. Various alternative interpretations of the relationship between the vacuolar osmotic pressure and cold resistance have been advanced, but none is supported by adequate experimental data. In conclusion, there is reason to believe that resistance is related more to the properties of the protoplasm than directly to those of the vacuolar sap, as has been pointed out by Kramer (1955).

The ability of plants to grow in saline and desert environments is directly related to their capacity for holding water within the cell and thus to maintain higher osmotic values in the cell than in the soil solution. In most plants which have been studied, both under experimental conditions and in nature, osmotic pressures are significantly higher than those of the soil solution in which the plants grow. Plants are frequently ten or more atmospheres higher than the soil solution (Eaton, 1943; Walter and Steiner, 1936).

To the already suggested roles which vacuoles and their contents play in the physiology of the plant may be added functions in cell division and cell elongation. The role of the vacuoles in cell division has been shown to be of importance in a number of plant tissues. Castetter (1925) studied cytokinesis at pollen cell formation in the legume *Melilotus alba*. He observed that following meiosis, numerous small vacuoles form, increase in volume and fuse together in such a manner as to block out the cytoplasm into four separate nucleated masses, which become the four cells of the tetrad. Bailey (1954) demonstrated the importance of vacuoles in the distribution of the cytoplasm in the long, tapered cambial-initial cells. Harper (1914) and Harper and Dodge (1914) showed that the large masses of cytoplasm in certain fungi are subdivided by the activity of vacuoles during spore formation.

Cell elongation is a process of the greatest importance to plants and

has been extensively studied and discussed (cf. Burström, 1961; Frey-Wyssling, 1950; Thimann, 1951; Went and Thimann, 1937). It occurs in innumerable physiological processes, including geo-, photo-, and thigmotropism, growth of leaves, including the uncoiling and elongation of fern fronds, the elongation of shoot and roots, of root hairs, stamens, etc. Moreover, the rate at which cell elongation occurs can be quite remarkable. The seed hair cells of cotton may increase about a thousandfold in length, from about 28 μ to about 28,000 μ, in 15 days. These cells grow at a rate of 78 μ per hour. Similarly, epidermal cells of oat coleoptiles grow at a rate of 21 μ per hour (cf. Ziegler, 1961).

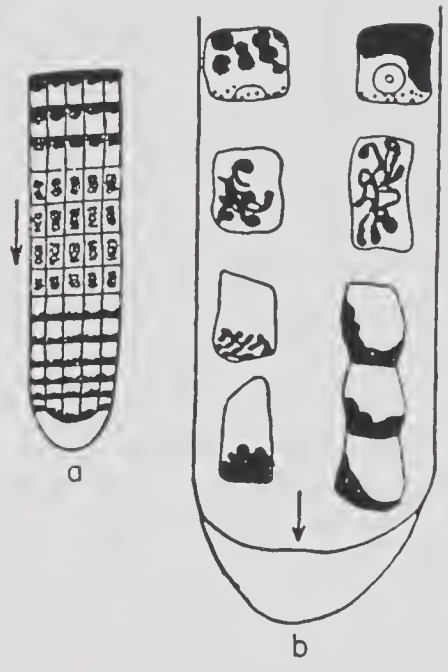

FIG. 27. Barley roots vitally stained with neutral red and then centrifuged. The chondriosome-shaped vacuoles of the meristem, heavier than the cytoplasm, are displaced centrifugally; those taking in water in the region of differentiation are not affected; those still higher in the root, lighter than cytoplasm, are displaced centripetally. From Milovidov (1930).

As recognized by de Vries (1877a, b), the elongation of cells is based upon two processes, the osmotic uptake of water by the vacuole and the stretching of the cell wall. The physics and chemistry of the wall stretching have been thoroughly discussed in Volume 14 of Ruhland's (1961) "Encyclopedia of Plant Physiology" and will not be considered here. The interrelationship of the processes of water uptake and cell elongation may be recognized from the formula for the osmotic state of the cell. In simplest form the osmotic relations may be described by:

$$S = O - (W + A)$$

where S is the "suction pressure" or "diffusion pressure deficit" (DPD) of the cell, O is the osmotic pressure of the cell, W is the wall pressure against the cell contents, and A is the pressure of factors outside the cell upon it. The "turgor pressure" of the cell is equal to $(W + A)$.

The turgor pressure, which tends to stretch the cell wall, characteristically amounts to several atmospheres of pressure. As the young, plastic wall is stretched during the osmotic uptake of water by the vacuole, the cell sap becomes diluted and the osmotic pressure simultaneously decreases (cf. Burström, 1961; Hackett, 1952). The changes which take place in the cell wall during cell growth have been described by Mühlethaler (1961). As would be expected, the cells of tissues placed in isosmotic solutions do not elongate (cf. Ordin *et al.*, 1956).

Figure 27 indicates the positions assumed by vacuoles within cells located in various developmental zones of centrifuged barley roots. The denser vacuoles of meristematic cells at the root tip are found at the "bottom" of cells. In elongating cells, as their contents become diluted and cell-sap specific gravity decreases, the vacuoles assume a central position and finally are found at the top of cells which have elongated.

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and Biesele, 1956) produce similar effects: a speeding of mitochondrial movement and an exaggeration of their end-to-end fusion so that the cell comes to possess a few very long filaments. Frederic (1958) finds support from his extensive investigations for two important views: (1) that mitochondrial substance is in dynamic equilibrium with submicroscopic cytoplasmic substance ("la substance fondamentale") (Fig. 2), and (2) that mitochondria possess two types of movement, an active

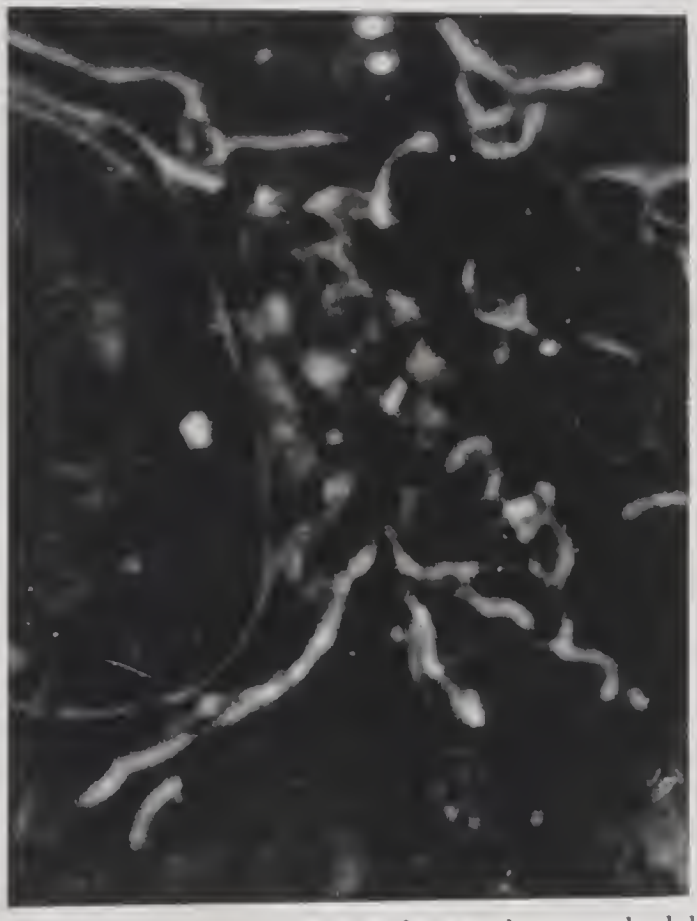

FIG. 2. Living chick fibroblast in tissue culture, photographed by Anoptral phase contrast microscopy. To the right of the nuclear membrane is the Golgi zone, in which "la substance fondamentale" and refractile droplets are present. Magnification: × 3000. From Frederic (1958).

one in which they change their form, and a passive one by which they are moved about in the cell.

In a later section (page 358) we shall again discuss the fate of mitochondria during division of cells in culture. Here we will note that in some instances the plasticity of mitochondria is dramatically displayed. They round out and become much smaller and, when division is complete, they revert to their original size and shape. Whether any disappear entirely, to have new ones reappear after division, cannot be established by light microscopy because of its limited resolving power.

The study of living cells cannot be expected to answer such questions as: what are the mechanisms by which mitochondrial number is kept relatively constant although they are not seen to divide in mitosis?; is mitochondrial movement passive or active? (see Tobioka and Biesele, 1956; Frederic, 1958); what is the functional meaning of mitochondrial movement, or their apparent contacts with the nucleus?; is there a submicroscopic mitochondrial "material" or "substance fondamentale" with which the mitochondria are in equilibrium? (Frederic, 1958).

Some caution concerning observations on tissue culture cells may not be out of order. Wilson (1928) long ago suggested that such cells may be living under stress. They may be demonstrating capacities never exhibited in the intact organism where they are influenced by neural and hormonal factors and probably directly by adjacent cells. Thus, some cells undergo "abnormal" chromosomal changes in tissue culture (Hsu, 1959). Many cells show extensive surface bubbling at late anaphase and telophase, which Barer and Joseph (1956) have shown can be prevented, in insect spermatocytes, when the cells are constrained by a mass of neighboring cells and spermatid fibrils. Because of their sensitivity, mitochondria might well be exhibiting "abnormal" behavior when fragmenting or fusing. It will be of great interest to learn how mitochondria behave in cells (human and opossum) grown under more optimal conditions in which the chromosomes remain stable for hundreds of generations (Tjio and Puck, 1958). Naturally, such concern about the effect of stress of mitochondrial behavior does not apply to the same extent to observations of mitochondrial movements in living Protozoa (Fauré-Fremiet, 1910), ova, or certain plant tissues. Guilliermond (1941) has described changing mitochondrial shapes, thickenings, and branchings in motion pictures of cells from a variety of fungi and higher plants. Sorokin (1941) described mitochondrial division in plant cells.

Not all cells need be alike in the extent of mitochondrial plasticity. In mammalian cardiac muscle or kidney tubule cells, in snail spermatozoa, and in other cells the mitochondria are probably firmly anchored, but in others they appear to be free to move about. Palade (1956a) has suggested that when mitochondria move through a cell they may have better access to substrates used in oxidative phosphorylation and other biochemical processes, and when they are more firmly situated they probably have important relationships to cell membranes or intracellular areas of specialized activity.

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CHAPTER 5

Sensory Cells

By R. CORDIER

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I. Introduction

Sensory receptors of Metazoa present an extraordinary variety of anatomical structures. The maintenanee of suitable eonditions of life of the animal is conditioned by its capaeity to react adequately to varying conditions both of the environment and of the inner milieu. The animal organism is therefore endowed with multiple sensory organs, extremely polymorphic from an anatomical or histological view point and having the samc general function: to perceive external or internal stimuli of all kinds and to transfer the energy thus received to reacting organs, muscles, or glands, sometimes immediately, as in lower Metazoa, more generally by means of a nervous impulse transmitted by an intermediary tissue, the nervous tissue.

The multiplicity of stimuli on the one hand, the extreme variety of structure on the other hand, has lead the biologist to a very tempting

concept, according to which the different kinds of known receptors are specifically differentiated and endowed with a specific discriminating sensitivity for different kinds of stimuli. This concept may be true in many cases. However, its accurateness depends essentially on the possibility of physiological analysis of the quality of the stimulus registered. This can be done with reasonable reliance in the case of human sensory organs, or of organs of animals having the same anatomical and histological structure as the human. Even in this case, serious doubts have recently been emitted on the specificity of cutaneous receptors for touch, pain, or thermal perception. How much more difficult will be the problem when sensory organs not present in the human are considered, as, for instance, the manifold sensory cell types of insects, the ampullae of Lorenzini of sharks and skates, the great variety of nerve endings belonging to the lateral-line system of lower vertebrates? How will the human mind grasp the importance and the nature of sensory information coming from organs lacking in human anatomy? However, this problem, though certainly highly difficult, has been approached with success by means of indirect methods, such as the observation of associated reflexes, or by physiological methods, especially the recording of the electrical activity of afferent nerves after appropriate stimulation of the end organ.

In a morphological survey of the very diverse aspects of sensory activity, a clear distinction should be made between the concept of sense organ and that of sensory cells. The concept of sensory cell implies a more or less highly differentiated cell, endowed with special cytologic features related with its function, i.e., the perception of the stimuli. However a great number of sense organs do not have such cells; for the fulfillment of their sensory function, they make use of otherwise differentiated cells, such as epithelial cells, muscle cells, connective or Schwann cells.

The essential and necessary feature is the existence of an afferent nerve that will lead the sensory impulse to the nerve centers.

On the basis of these fundamental differences, a classification of sensory cells of vertebrates can be proposed. The extreme variety of sensory cell forms in invertebrates will not be included in this chapter.

A. Types of Sensory Receptors

1. Sensory Receptors of the First Type

In this type, the true primary sensory cell is the ganglionar neuron, uni- or bipolar, located in the cranial or spinal ganglia and sending its dendrite peripherally as afferent nerve fiber. The afferent

dendrite is either a free ending, or is in contact with nonnervous cells, which cannot be considered as true specialized sensory cells but nevertheless are essential for the transmission of the stimulus; such are the muscle cells in stretch receptors, the laminate cells surrounding the dendritic terminal in the different varieties of dermal corpuscles (Pacini, Meissner, Herbst), the large "tactile" cells of the corpuscle of Grandry, the "tactile" cells of Merkel. Such cells can be considered as sensory by adaptation, although their true receptory function and their role in the transmission of the stimuli remains highly dubious. They could be better referred to as "pseudosensory" cells (sensory cells *largo sensu*).

Many of these organs are highly specialized, and able to transmit very discriminating sensations. They constitute a favorite object of study, both for histologists and physiologists. However, in spite of their functional importance, they are not endowed with the kind of specialized cells that a cytologist could consider as true sensory cells. They should therefore not be included in the present chapter.

However, some of them have been the object of recent investigations bearing on their histochemical aspects or on their fine structure or on their physiological properties; two examples of such efficient organs will be referred to, chosen for their highly differentiated histological structure and the high degree of their innervation. The organs referred to will be the organ of Eimer in the snout of the mole, and the pit organ of pit vipers.

2. *Sensory Receptors of the Second Type*

This group includes sense organs endowed with highly specialized differentiated cells, having in common some characteristic cytological features. (a) The sensory cells are disposed at a free surface (inner or outer), and their apical pole, being in direct contact with the stimulating agent, presents some differentiated structure, which can be a more or less modified cilium or a cuticular formation ("sensory hair"), or a number of microvilli. (b) The cytoplasm of the sensory cells generally contains submicroscopic vesicles very similar to synaptic vesicles found in presynaptic position in efferent nerve fibers.

3. *Sensory Receptors with Neurosensory Cells*

Finally, in some cases there exists, mainly in invertebrates, and in a lesser degree also in vertebrates, a type of sensory organ where the specific cells combine the nature and properties of true sensory cells, with apical differentiated pole, and those of a nerve cell, being themselves the origin of the afferent nerve fibers that transmit the sensory

information to the brain centers. Such cells are termed neurosensory cells, the best-known example in vertebrates being the olfactory cell.

B. Classification

The detailed cytological knowledge of sensory or neurosensory cells is actually still very incomplete. Abundant and exhaustive observations with the light microscope have brought a very satisfying understanding of the histological structure of numerous sense organs in vertebrates. On the other hand, electron microscope observations have been accumulating in recent years; for obvious reasons, they are still restricted to a limited number of organs or animal species, for instance, auditory and vestibular cells in rodents, gustatory cells in some mammals. Finally, some information has been obtained concerning the histochemical aspects of sensory organs, mainly in gustatory and olfactory organs, and in the cutaneous nerve endings of first type.

A rational classification of sense organs is not easy. A physiological basis could be used, classifying the organs according to their function, as tangoreceptors, pressoreceptors, chemoreceptors, thermoreceptors, receptors of vibratory or electric energy. However the strict specificity of many receptors for a given type of stimulation is by no means certain and, even in mammals, has been seriously challenged by modern authors, e.g., Weddell. In sense organs of lower vertebrates, as in the ampullae of Lorenzini, physiological research has pointed to a receptivity for mechanical, thermal, and electrical stimulation. Perhaps evolution has lead in higher vertebrates to a greater specificity, e.g., auditory cells; nevertheless, in other cases, as in the skin receptors of mammals and man, the existence of specific and different receptors for sensations of touch, heat, cold, pain, etc., is still doubtful. Moreover, specificity of the receptor must be understood within reasonable quantitative limits of the stimulus. It is a matter of fact, for example, that acoustic stimuli reaching a high intensity elicit a painful sensation in addition to the acoustic one. Generally speaking, any form of sensory energy which attains a noxive level, can provoke nonspecific sensations leading to nociceptive reflexes.

A classification on a histological morphological basis seems therefore more adequate. Following the general considerations set forth before, we proposed to adopt the following plan:

I. Neurosensory cells
   - A. Olfactory cells
   - B. Sensory cells of the organ of Jacobson
   - C. Ependymal sensory cells

II. True sensory cells (sensory cells *stricto sensu*)
  A. For special visceral sensitivity: gustatory cells
  B. For special somatic sensitivity
    1. The lateral-line system
    2. The labyrinthine sensory epithelia

III. Pseudosensory cells (sensory cells *largo sensu*)
  A. In the organ of Eimer of the mole
  B. In the pit organ of pit vipers

II. Neurosensory Cells

A. Olfactory Mucosa

In all orders of vertebrates the olfactory mucosa constitutes a more or less important part of the nasal cavity, though its extension and localization varies greatly. Generally the olfactory epithelium occupies the more dorsal part of this cavity, but it can descend more or less downward, abutting at the level where it is replaced by respiratory mucociliated epithelium. Very often (Amphibia, Chelonia) alternate regions of olfactory and respiratory epithelium form the lining of the nasal fossa. In fishes and urodeles, this alternating disposition is very regular and the olfactory portion is represented by a series of olfactory buds, separated by small strands of respiratory epithelium and reminiscent of taste buds.

The olfactory mucosa is formed by three kinds of cells: (1) olfactory cells; (2) sustentacular cells; (3) basal cells.

1. Olfactory Cells

The olfactory cell is a primary sensory element or neurosensory cell, i.e., it is both the sensory peripheral receptor and the origin of the olfactory fibers. Olfactory cells, in all orders of vertebrates, are elongated slender elements, usually superposed in several layers in the thick epithelium; their broadest part contains the nucleus; the apical part tapers in the direction of the surface, where it protrudes through the limiting membrane and ends with a globular expansion, the olfactory rod (olfactory vesicle), bearing a small number of cilia implanted on basal bodies (Figs. 1–3). This apical protrusion is easily identified in all classes of vertebrates, though the number and the length of the cilia are variable. In teleosts, amphibians, and reptiles, the cilia are very long, reaching sometimes more than 100 μ (Fig. 4). In birds and mammals they seem to be much shorter and are not easily seen with the light microscope. Finally, it must be emphasized that the olfactory vesicles are by no means uniform in length and thickness (Fig. 1) and

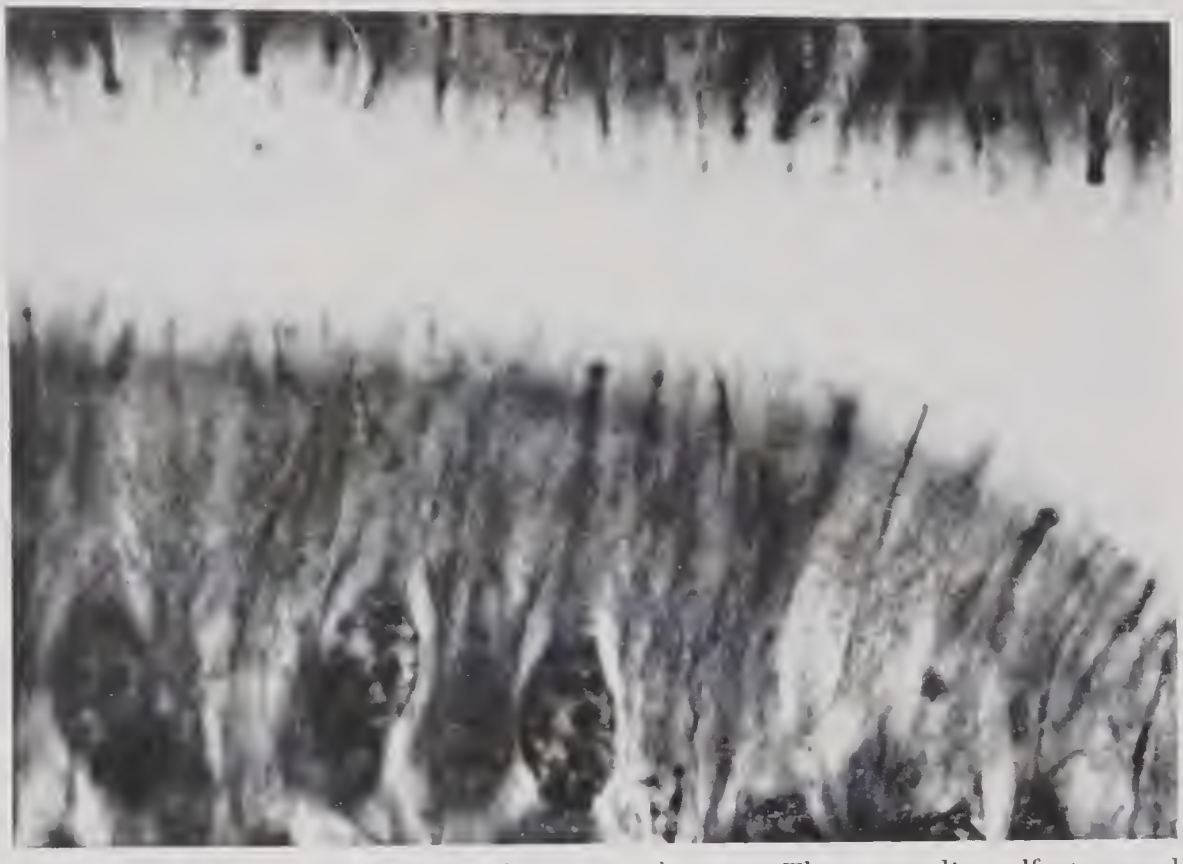

FIG. 1. Olfactory epithelium of *Myxine glutinosa*. The protruding olfactory rods present great variations in thickness and in length.

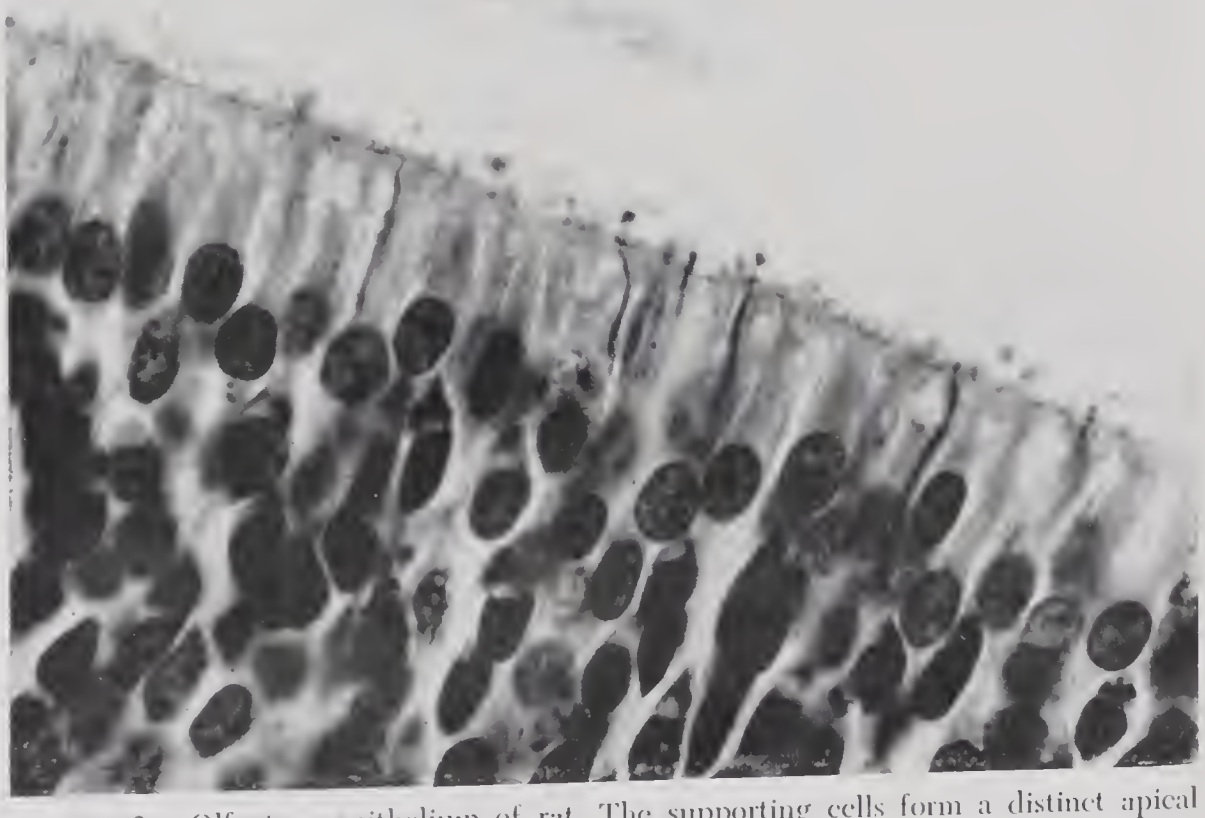

FIG. 2. Olfactory epithelium of rat. The supporting cells form a distinct apical limiting membrane. Apical extremities of olfactory cells with protruding olfactory vesicles of different length and thickness.

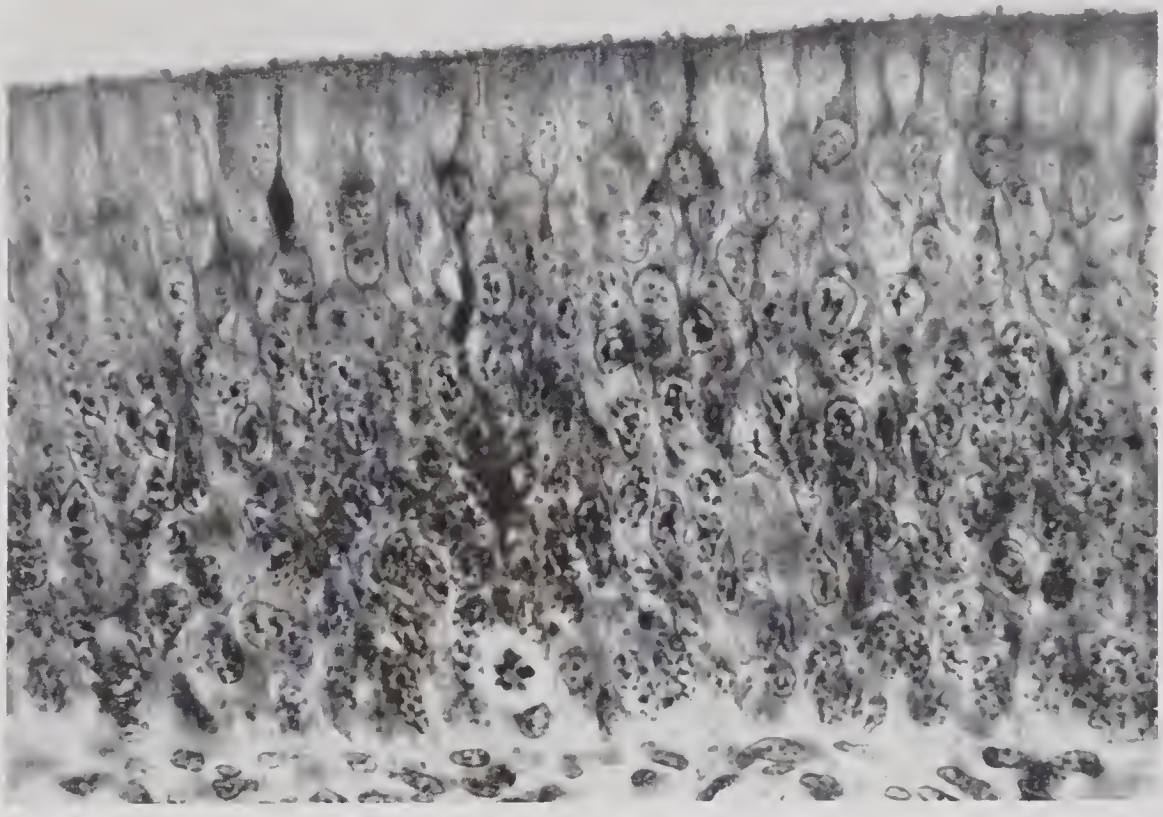

FIG. 3. Olfactory epithelium of embryo of opossum. Olfactory cells with protruding olfactory vesicles are forming in the upper layer; undifferentiated cells in the lower regions.

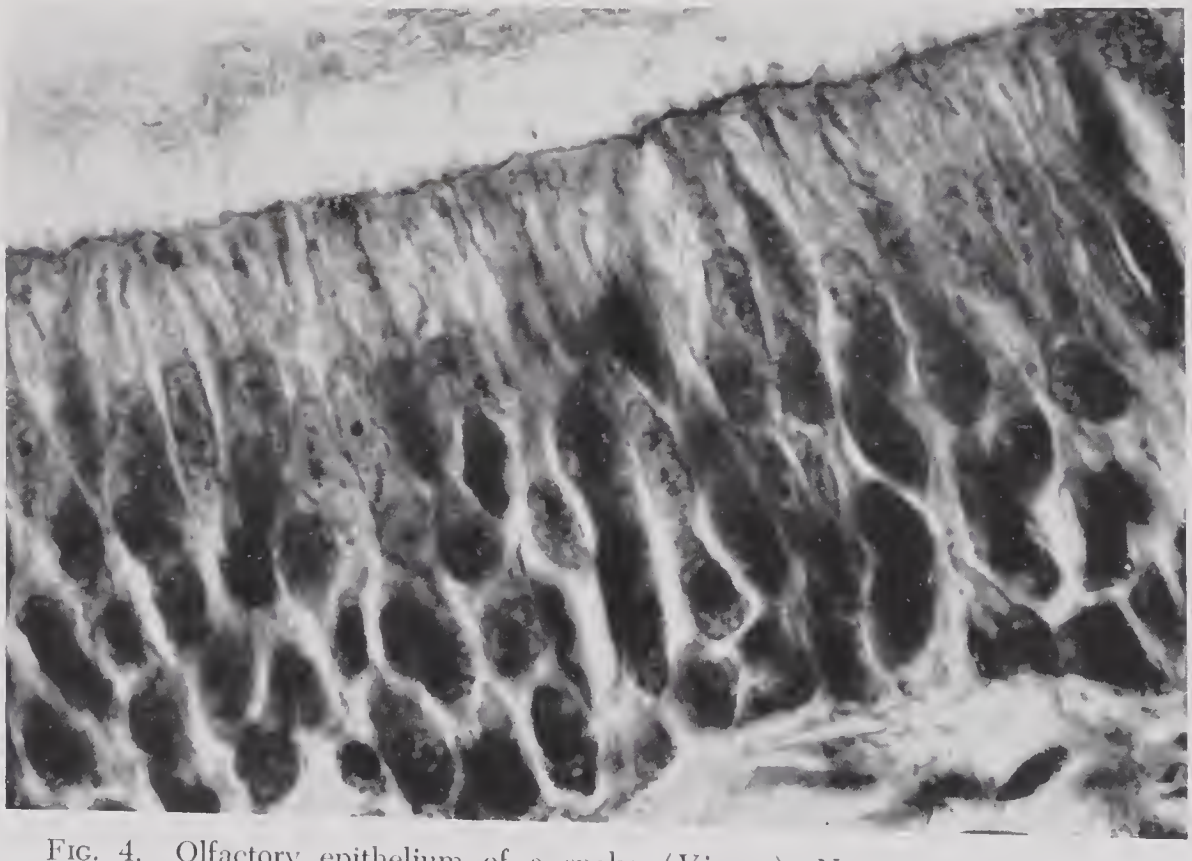

FIG. 4. Olfactory epithelium of a snake (*Vipera*). Numerous small olfactory vesicles with long cilia.

that, therefore, a great variety of sensory cell forms exist in one olfactory mucosa. In most cases the olfactory rod is not free but included in a mucinous layer covering the surface of the mucosa. This is the case at least in all airbreathing species. It seems therefore that olfactory sensation necessitates the previous dissolution of the odoriferous substance in the slime layer, so that finally all olfaction takes place in a fluid milieu. However, when the cilia are very long, they definitely extend through this mucous layer and are free in the nasal cavity. In aquatic species, it is generally considered that this slime layer is absent, the nasal cavity being filled with water.

Electron microscopy observations have demonstrated the cilium nature of the olfactory rod. de Lorenzo (1957), Bloom (1954), and Bloom and Engström (1952) stated that the olfactory vesicle is a cytoplasmic projection filled with small vesicles and bearing characteristic cilia with 9 protofibrils or groups of protofibrils. Basal bodies could be seen, from which emanated slender filaments, occasionally cross striated. de Lorenzo, in the young rabbit, rarely found more than 3 cilia per cell whereas Bloom in the toad, could observe 6–12 cilia, some of them very long and reaching the mucinous covering of the mucosa, where they joined in a netlike formation.

From the basal pole of the olfactory cell arises a very fine and slender filament which has the value of an axon and is very difficult to follow with the methods of classical light microscopy. However Golgi impregnation reveals its course. The basal filaments pass into the subepithelial connective tissue, where they form the fila olfactoria. The neuroepithelial nature of the olfactory cells is further evident from the presence of neurofibrils and, occasionally, inclusions resembling Nissl bodies.

The olfactory epithelium can be vitally stained; trypan blue, introduced into the nasal cavity after a few minutes impregnates the olfactory cells, the staining starting with the apical pole (Seki, 1941). Some authors hold that the penetration of the dye through the epithelium is effected by means of narrow spaces in communication with the surface at one side and with the lymphatic sheaths of the fila olfactoria at the other side. It has been demonstrated, moreover, that the same dye introduced into the cerebrospinal fluid rapidly reaches the olfactory mucosa and oozes into the nasal cavity.

2. *Sustentacular Cells*

Sustentacular cells are more numerous than olfactory cells. They are cylindrical in shape, their apical poles adjust in forming a limiting mem-

brane, reinforced by terminal bars and intercellular cement; the olfactory rods of the sensory cells protrude through this membrane at regular intervals, this disposition being clearly observable in tangential sections. In mammals the supporting cells do not possess apical cilia, their outer surface being covered with microvilli visible with the electron microscope. In Amphibia, however, long and short cilia can be observed in freshly dissociated material (Hopkins, 1926).

Some observations bearing on the histochemical aspect of the olfactory epithelium should be noted. According to Baradi and Bourne (1953), esterase and phosphatase can be demonstrated in the body of the olfactory cells, but are absent at the apical pole and in the olfactory vesicles.

In mammals, the olfactory mucosa has generally a yellowish tint which can be attributed to the presence, in the apical region of the supporting cells, of brownish or yellowish granules, that contain a fat-soluble pigment adsorbed on fatty inclusions. Gerebtzoff (1953) found a positive plasmal reaction. With respect to function, it seems that for the time being no definite relation can be established between the cytological structure and the chemoreceptory function of the olfactory cell. It is possible that different cell types, which differ by the volume of the olfactory vesicle or the number of cilia, could be held responsible for functional olfactory discrimination. On the other hand it has been claimed (Gerebtzoff, 1953) that some parallelism exists between the intensity of the yellowish coloration of the mucosa and the acuity of olfactory perception. However it is generally admitted that the yellow pigment is present in the supporting cells, not in the sensory elements.

3. *Basal Cells*

The presence of basal cells in the olfactory mucosa has often been claimed. Such cells, with scanty cytoplasm and a large nucleus, could be the origin of new supporting cells.

B. Sensory Cells of the Organ of Jacobson

The organ of Jacobson or vomeronasal organ is a specialized part of the nasal cavity. Its morphology, development, and nervous connections have been widely studied throughout the entire vertebrate phylum. Its precise function, however, remains still dubious.

In many orders of mammals, the vomeronasal organ is present in the adult stage (Marsupialia, Insectivora, Rodentia, Carnivora, Ungulata) as an elongated tubular formation located on both sides of the nasal sagittal septum. Its anterior end opens either into the nasopalatine canal

of Stenson, or directly in the nasal cavity. Its posterior end is closed. On transverse section of the head, the organ appears as a crescent-shaped hollow epithelial formation, its lumen being invaginated from the lateral side by connective or vascular tissue. Often a cartilaginous groove supports the soft tissues. The mesial wall of the tube is thickened, in sharp contrast with the lateral wall, which is much thinner. In *Talpa*, however, the outline of the vomeronasal organ is regular, round or oval. Entirely absent in birds, the organ of Jacobson is highly developed in

FIG. 5. Transverse section of the head of a snake (*Vipera*) showing the voluminous organs of Jacobson, opening into the mouth (left side of figure).

snakes and lizards. Here it appears as a globular epithelial mass, very conspicuous in snakes (Fig. 5), entirely independent from the nasal cavity and opening by a short canal directly into the mouth. The dorsal and lateral walls of the organ are covered by thick sensory epithelium, while its ventral wall bulges into the cavity in consequence of the development of vascular tissue and is covered by a thin, nonsensory epithelial layer. In Chelonia no independent vomeronasal organ exists, but a specialized ventrolateral part of the nasal cavity has been homologized with it by several authors. In Amphibia a comparable situation is found: an independent organ of Jacobson is lacking, but there exists a ventral flattened expansion of the nasal cavity partly covered with

sensory epithelium. In Urodela, the lateral part of this outgrowth could be the equivalent of the vomeronasal organ, whereas in Anura the medial region of this same ventral diverticulum has been considered as representing the organ of higher vertebrates, in a corresponding anatomical location.

No organ of Jacobson has been found in fishes.

1. Histology

Although the general morphological features of the vomeronasal organ have been the object of extensive research, a detailed description of its cytology is rarely to be found. In well-developed stages of the organ, as in many adult mammals, the sensory epithelium occupies the inner wall, while the outer wall is covered by ordinary respiratory epithelium. In the mole, however, sensory cells are present throughout the whole covering epithelium, mixed with respiratory cell types.

It is generally claimed, by numerous histologists, that the vomeronasal sensory epithelium is identical with the olfactory mucosa. Kolmer (1927) states that the "sensory cells are high, slender elements, which are similar to the olfactory cells, though less completely developed." Matthes (1934) refers to "a sensory epithelium which in all essential features, corresponds to the olfactory epithelium." Zuckerkandl (1910) in a monograph on the organ of Jacobson makes an identical statement. Klein (1881) and Read (1908) found the vomeronasal epithelium very similar to the neuroepithelium of the olfactory mucosa. According to Addison and Rademaker (1927) the thicker Jacobsonian epithelium conforms in general to the olfactory neuroepithelial type.

Throughout these different opinions appears a definite doubt on the cytologic identity between nasal and Jacobsonian sensory epithelium since all authors refer to general similarities or differences in development. It is therefore the more surprising that the exact cytology of the vomeronasal organ has not been better investigated.

A careful examination of this structure, either in mammals (opossum, rabbit, rat, hedgehog) or in snakes (*Vipera*, *Agkistrodon*) on well-fixed material shows a very conspicuous deviation from the nasal olfactory epithelium.

In the vomeronasal sensory epithelium two cellular types can be distinguished, sensory cells and sustentacular cells. The sustentacular cells are prismatic elements with nuclei forming a regular layer in the upper part of the epithelium. Their apical elongated poles join together in a limiting membrane, the cell limits being there reinforced with

terminal bars. The sensory cells are present in multiple superposed layers; their nuclei are larger and stain lighter, and they occupy the lower two-thirds of the total height of the epithelium. Their apical pole tapers in a threadlike cytoplasmic strand, enlarging in a terminal bulb. However, this terminal bulb in no case protrudes beyond the limiting membrane, remaining beneath it. Neither apical protrusion nor cilia exist. Figures 6 and 7 show clearly this disposition in the rat and

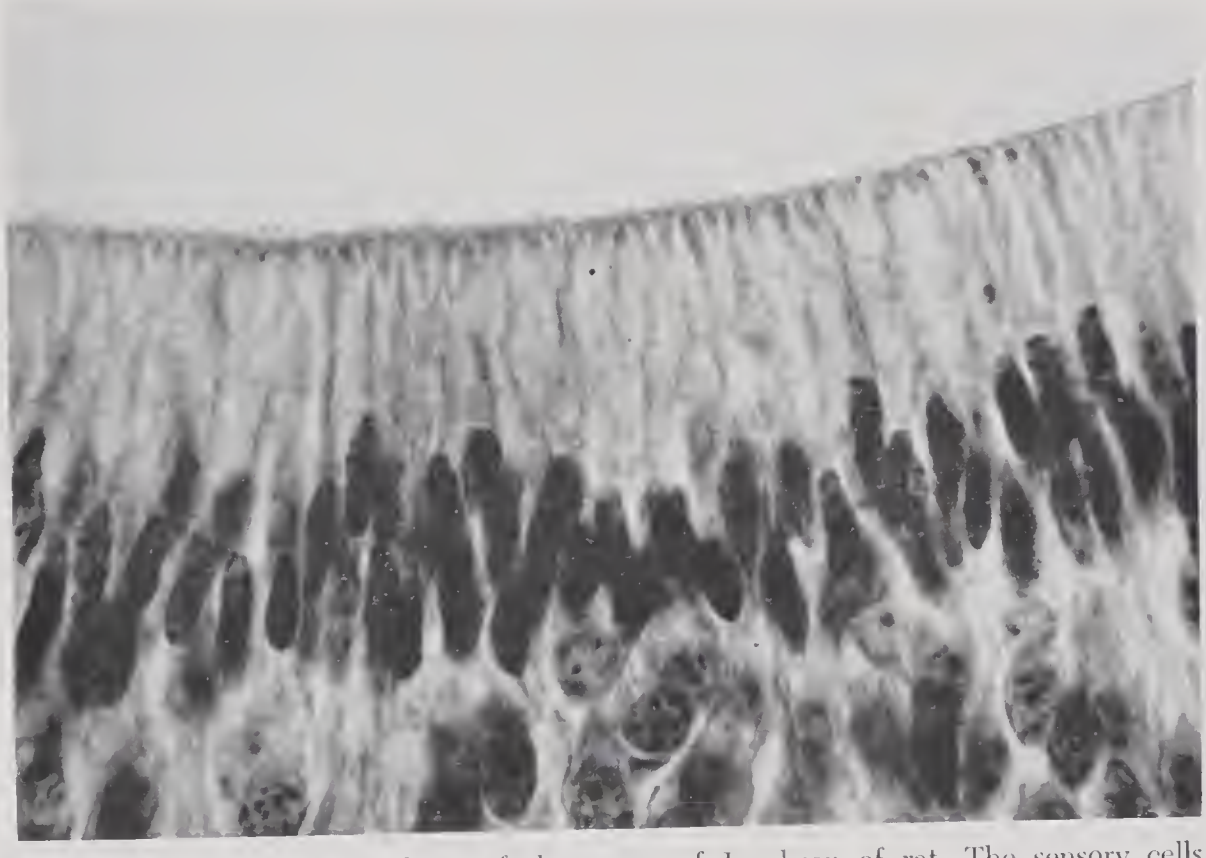

FIG. 6. Sensory epithelium of the organ of Jacobson of rat. The sensory cells end in clublike swellings that do not protrude through the superficial limiting membrane (compare with Fig. 2).

opossum, in contrast with Figs. 2 and 3 from the same animals, respectively. At the base of the epithelium large cells with vesicular nuclei can be found, recalling the structure of a ganglionic nerve cell. In young *Erinaceus*, it is very easy to follow the migration of these cells along the vomeronasal nerves, where they form, at irregular intervals, small ganglionic masses.

In snakes an identical cytological difference exists between olfactory and Jacobsonian epithelium. In the vomeronasal organ the supporting cells form a limiting membrane beneath which can be seen numerous clublike endings abutting on this membrane and representing the taper-

ing apical extremities of the abundant sensory cells, which form the bulk of the organ and are grouped in epithelial masses separated by richly vascularized connective tissue.

Electron microscopic observations on the vomeronasal organ are lacking; they are highly desirable in order to appreciate the submicroscopic difference between its sensory cells and true olfactory sense cells.

The function of the organ of Jacobson remains somewhat obscure.

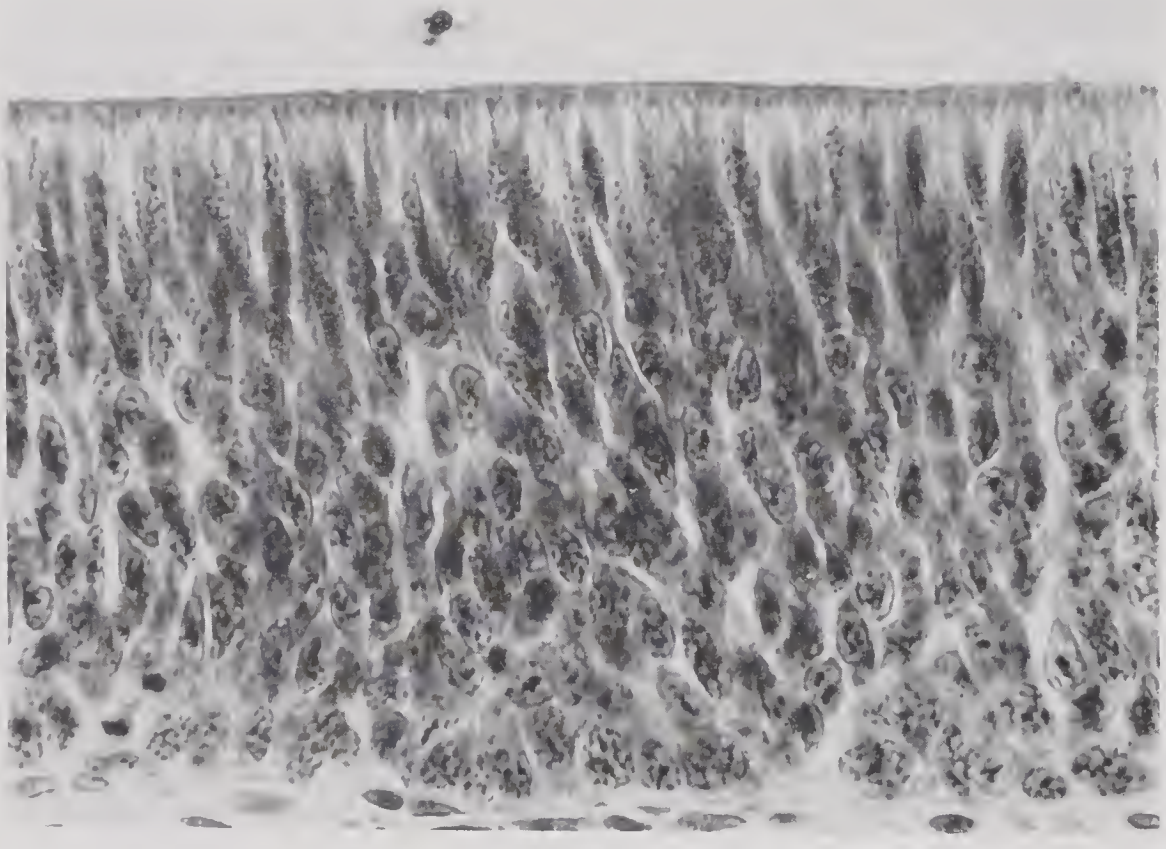

FIG. 7. Sensory epithelium of the organ of Jacobson in embryo of opossum; clublike endings of sensory cells, beneath the limiting membrane (compare with Fig. 3).

Kolmer's statement according to which the Jacobsonian sensory cells are "insufficiently developed" implies that the vomeronasal organ, with respect to function, must be considered as less important than the olfactory mucosa. However, several authors attribute to the organ a not negligible part in the appreciation of the olfactory qualities of the food. In mammals, experimental research (Broman, 1920) has shown that normally the organ of Jacobson is filled with fluid, not with air. This is the result of a pumping action, exerted by the vascular, erectile tissue in the organ on fluid present in the nose, or in the mouth, or in both. In serpents it has been stated that the contents of the oral cavity bearing

olfactory stimuli are conveyed to the organ of Jacobson by means of the extremities of the tongue.

The important innervation of the organ and its relation with olfactory centers in the brain lead to consider the organ of Jacobson as the anatomical receptor of a specialized olfactory sense. Bromann has suggested that in macrosmatic animals having a tracking sense, the vomeronasal organ is important as a tracking organ or a mouth smelling organ, and represents the water olfactory organ of lower vertebrates, altered and adapted for life on land.

C. Ependymal Sensory Cells

The ependymal lining of the cerebral ventricles consists of high cylindrical cells with differentiated apical and basal poles and considered to be a modality of glial tissue. In several regions however, the ependymal cells can present a very high degree of morphological and functional differentiation and acquire the cytological characteristics of sensory elements. The highest degree of this modification is of course attained in the photoreceptor cell; indeed the morphogenesis of the retina reveals very clearly that the visual cells, with their cones or rods take origin from the inner invaginated sheet of the optic vesicle and are thus of ependymal origin, exactly as the pigmented retinal epithelium which takes origin from the outer sheet. The cytology of the photoreceptor cells is described elsewhere in this treatise (cf. Chapter 6 of Volume IV) and will not be referred to here. However, it must be remembered that electron microscope observation has clearly demonstrated that the photoreceptor part of the visual cells, i.e., the rod or the cone, is a modified cilium and that the characteristic picture with 9 protofibrils can be seen at the junction of the outer and the inner segments.

Other differentiations of the ependyma are known, although their functional significance cannot be compared with that of the visual receptors and remains, moreover, uncertain and open to discussion. We will refer to the two principal ependymal regions where a sensory function has been suggested: (1) the saccus vasculosus, and (2) the epiphysis.

1. The Saccus Vasculosus

In Selachii and many orders of teleosts, the floor of the hypothalamus presents a more or less lobulated evagination, located rostrally to the hypophysis and very conspicuous at the ventral aspect of the brain for its abundant vascularization, justifying the denomination of saccus

vasculosus. The organ is hollow, and its branching lumen is covered with a highly differentiated ependymal epithelium, which, from a cytological point of view, offers the characteristic features of a sensory epithelium. It consists indeed of two types of cells: (*a*) sustentacular cells, and (*b*) the so-called coronet cells.

a. Sustentacular cells. These are slender ependymal cells with a narrow base; their apical pole enlarges and appears as an inverted

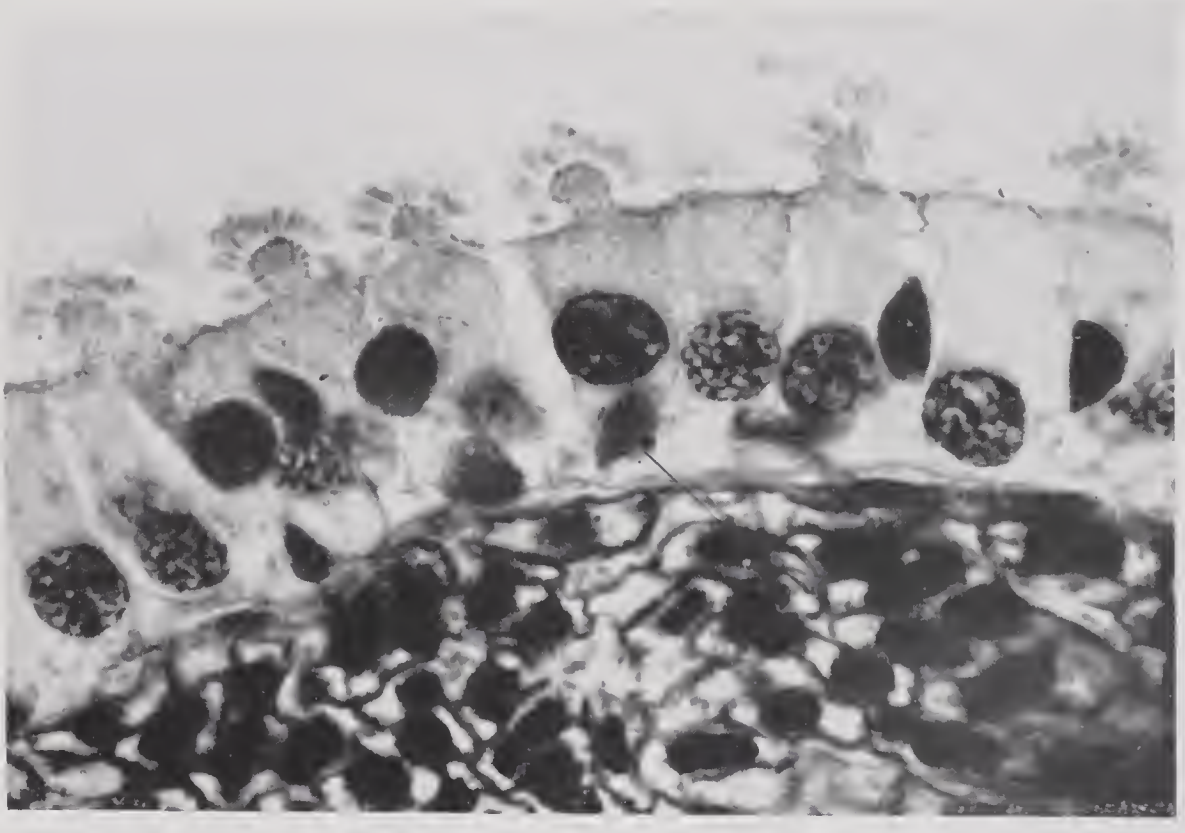

FIG. 8. Epithelium of the saccus vasculosus of the trout (*Salmo fario*). Coronet cells, with apical protrusions, basal bodies, and modified cilia. Supporting cells with ovoid nuclei.

conical or pyramidal structure, the nucleus being often located in this part, and thus very superficially.

b. Coronet cells. These cells are located between the sustentacular cells. They appear as cylindrical cells with a very characteristic apex (Fig. 8). The apical cytoplasm forms a superficial hemispherical protrusion, at the periphery of which a regular layer of basal bodies can easily be seen, each bearing a slender filament terminating in a globular expansion. This very interesting and unique cytological appearance, known as "coronet," is very similar to a pincushion furnished with regular radially implanted pins with globular heads. Careful observation on serially sectioned material shows that the basal pole gives rise directly

to a slender filament that penetrates into the subepithelial layer and has the structure of an amyelinated nerve fiber. Bundles of such nerve fibers can be followed to the hypothalamus. The histological and cytological aspects of the saccus vasculosus have been described very exhaustively in the monograph of Dorn (1955). The cytological features of the coronet cells point to their neurosensory nature, and many authors have considered them to be primary sensory cells, comparable to the olfactory cells (Boeke and Dammerman, 1910; Dammerman, 1910; Beccari, 1943). As to the physiological importance of the sacculus vasculosus considered as a sensory receptor, different hypotheses have been advanced. The primitive conception of Dammerman, according to which the saccus vaseulosus is able to give information on the blood pressure or oxygen amount of blood and therefore indirectly on the depth of the water level, is entirely hypothetical and not based on any experimental work. Besides, according to Hoefke (1955) the volume of the saccus vasculosus in deepwater fishes is not proportionally larger than in fishes living near the surface.

However, recent authors (Bargmann, 1954; Van de Kamer *et al.*, 1954, 1958, 1960; Jansen and Van de Kamer, 1961) do not accept the sensory nature of the coronet cells. Histochemical arguments seem to indicate rather a secretory function. Bargmann observed in several species of teleosts, a positive periodic acid-Schiff (PAS)-reaction in the terminal globules of the coronet hairs, and the presence of PAS-positive material, or Gomori-positive material in the lumen of the saccus vaseulosus. Van de Kamer obtained variable histochemical pictures in the saccus vaseulosus of the trout, after injection of pilocarpine. A crescent-shaped region on the border of the apical cytoplasmic protrusion shows a deeply positive glycogen reaction after 48 hours, but no glycogen could be demonstrated in the hairs or their globular extremities; however the latter are stained easily with aldehyde-fuchsin. Van de Kamer suggests that glycogen is elaborated in the apical protrusion and progressively transformed in acid mucopolysaccharides at the level of the globules. Cleavage products could penetrate into the cerebrospinal fluid and modify its osmotic pressure. However, it should be emphasized that only a few coronet cells have shown pictures suggestive of a secretory process. According to Dorn (1955), who found secretory droplets in the coronet cells and a stainable colloid in the lumen, the secretory activity of the coronet cells must be admitted, but does not furnish any proof against the sensory nature of the cell.

Electron microscopic observations made by Bargmann and Knoop (1955) on the coronet cells of different teleosts have demonstrated

clearly the cilium-like structure of the slender filament attaching the globular extremities to the apieal protrusion: the characteristic disposition of 9 filaments has been observed. Furthermore in the terminal globules, tubules and vesicles were found that eontained large vacuoles whieh eould possibly burst and expell their contents into the lumen. Although Bargmann eoncluded from his investigations that the coronet cells have a secretory funetion, his observations cannot fail to show clearly the resemblanee and homologies of the terminal globular processes of the coronet eells with the outer article of the retinal rod, mainly when the latter strueture is observed in development or in experimental degeneration owing to lack of vitamin A (de Robertis and Lasansky, 1961; Dowling and Gibbons, 1961). It seems therefore that reasonable evidenee exists for considering the coronet eells as sensory, or better neurosensory, eells, although their funetional importance and the nature of the stimulus remain still the object of mueh speculation. The demonstration of some seeretory aetivity does not formally contradiet this opinion, sinee coexistenee of a nervous and a glandular aetivity is by no means exeeptional.

2. Epiphyseal Sensory Cells

The roof of the dieneephalon, rostrally to the habenular region, develops typically in two dorsally directed expansions, the parietal organs. The rostral parietal organ, indieated in the embryonal state of nearly all vertebrates, rarely persists in the adult state; if it does, it eonstitutes the pineal eye, present in *Sphenodon* and lizards. The eaudal parietal organ develops in the epiphysis or pineal organ, present in all elasses of vertebrates.

In the epiphysis of Amphibia (Kelly and Van de Kamer, 1960) and of lizards (Steyn, 1960), clusters of sensory cells have been deseribed, both with histological and histoehemical methods and by means of the electron microseope. The general histologie strueture is very suggestive of a sensory organ. Indeed both sustentaeular cells and epiphyseal sensory eells have been found. The sustentaeular eells are slender eells, enlarged at their apieal pole, whieh form a limiting membrane eovered with numerous submieroseopie mierovilli. The sensory eells in lizards are very similar to those in the pineal eye; they show, at their apieal region, piled-up lamellar formations resembling elosely the strueture of the retinal rod. In *Rana* (Kelly and Van de Kamer, 1960) found the sensory eells provided with an elongated eell body loeated beneath the limiting membrane and a more or less developed apieal protrusion extending beyond the limiting membrane; in this protrusion an outer

and an inner article can be identified (Fig. 9). An ellipsoid is present in the inner segment having a fatty composition, whereas in the outer segment positive PAS and positive aldehyde-fuchsin staining can be obtained. At certain levels of the epiphyseal lining, chiefly on the roof of

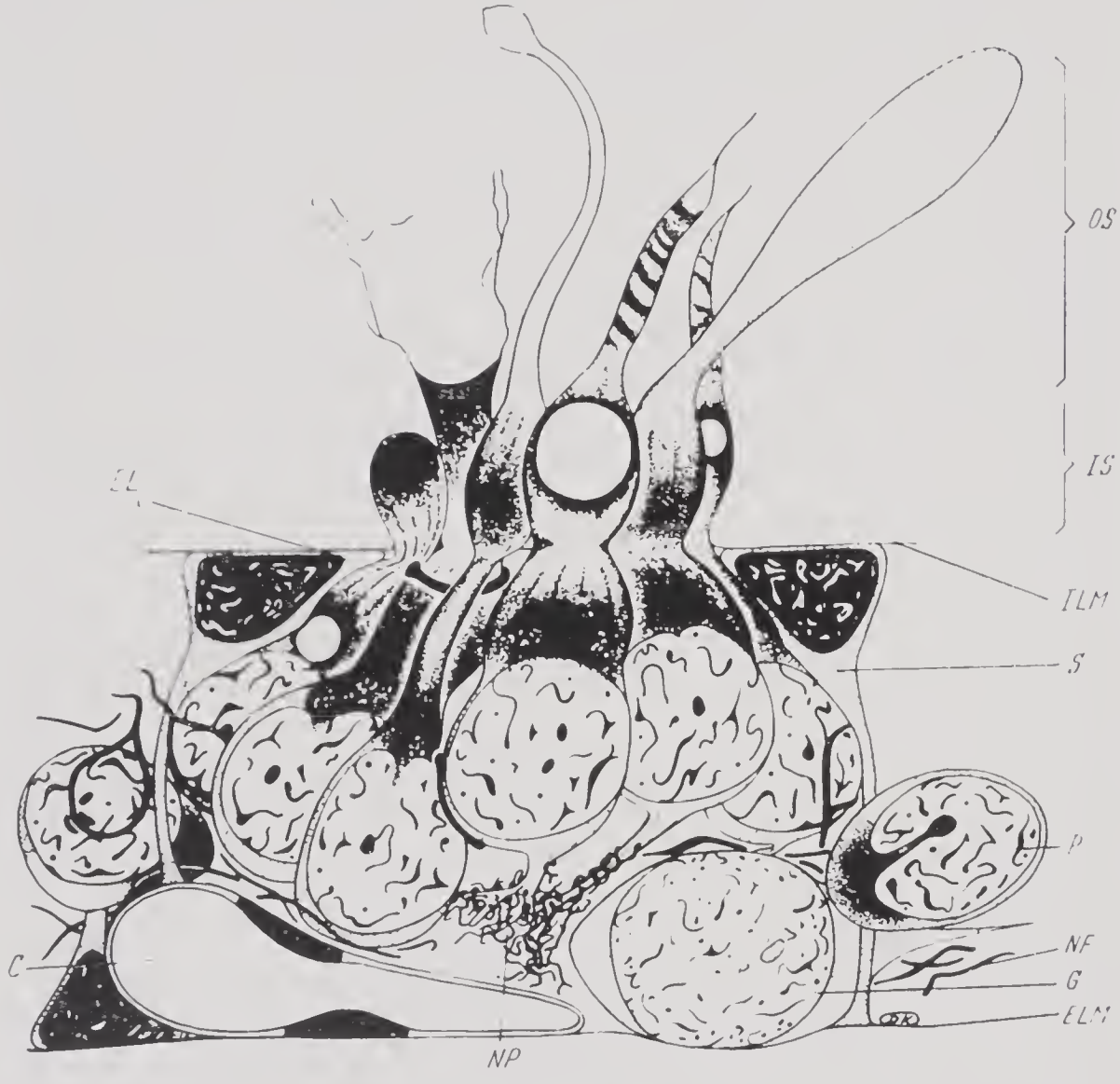

FIG. 9. Schematic drawing of epiphyseal cells of frog. *OS*, *IS*, outer and inner segments of apical protrusions; *ILM*, limiting membrane; *S*, supporting cell; *NF*, nerve fiber; *G*, ganglion cell; *NP*, basal process of sensory cell with neural plexus; *EL*, end loop of neural fiber. From Kelly and Van de Kamer (1960).

the epiphysis, the sensory cells possess basal nervous processes which seem to contact synaptically the dendrites of ganglion-like cells located deep in the epithelium.

Although the histochemical information seems to point to a possible secretory function, this fact cannot be invoked against the sensory nature

of the epiphyseal cell in lower vertebrates; the resemblance with photoreceptor is obvious, and even the histochemical data are similar to those obtained in retina. However, the cytological differentiation is less pronounced and seems rather abortive; nothing can be said about its functional importance. It has been suggested very pertinently (Steyn, 1960) that in lower vertebrates the hypothetic sensory role can be accepted, whereas in higher vertebrates (mammals, birds) a secretory function could dominate, although no precise information is available about the function of the organ.

III. True Sensory Cells (Sensory Cells *Stricto Sensu*)

A. Sensory Cells for Special Visceral Sensitivity: Gustatory Cells

Gustatory sensory organs are widely distributed among vertebrates, and, in association with olfactory receptors, constitute the most important chemoreceptors for finding and appreciation of food. They are known as taste buds, or neurogems.

A taste bud is a barrel-shaped intraepithelial cell complex, narrowing at its upper and lower parts while the central region bulges, corresponding to the location of the nuclei.

In mammals, taste buds are mainly situated on the tongue, in the various kinds of lingual papillae (papillae vallatae, foliatae, fungiformes) and are innervated by the IXth and VIIth cranial nerves. They can also be found, in small numbers, on the soft palate and the epiglottis, their number and distribution varying largely according to the species considered. In rodents and in insectivores (*Erinaceus*) the soft palate contains neurogems grouped in separate fields (Kaplick, 1953); no gustatory buds exist in this location in carnivores, ungulates, or man. In mammals, taste buds may be found on the free surface of the buccal lingual mucosa, their base protruding sometimes deeply into the dermis (e.g., soft palate of the rat); more often they are grouped in grooves formed by infolding epithelium, as in the papillae vallatae of the lingual V, or in the papillae foliatae at the side of the tongue. Gustatory sensitivity is restricted, in mammals, to the oral cavity. In lower vertebrates, mainly in several families of bony fishes (*Amia*, siluroids, cyprinoids), gustatory buds are widely spread in the outer skin, especially on the head, or at the surface of perioral barbels, when present (siluroid fishes). A special branch of the VIIth cranial nerve, the recurrent nerve of Weber, furnishes the innervation of the buds on the body.

In relation with the extensive development of gustatory sensitivity, the secondary taste centers in the visceroafferent zone of the oblongata

show a remarkable hypertrophy, appearing on the floor of the IVth ventricle as prominent structures known as lobus VII and lobus IX X.

In numerous species of Selachii, cutaneous sense endings have been described under different names, such as "Spaltorgane" "Spaltpapillen," "Grubenorgane," "eryptes sensorielles" (Petit and Budker, 1936). Sensory crypts are depressions of the skin, covered in sharks by special placoid scales whereas in skates they open at the surface by a narrow slit. The base of this depression is occupied by a sensory papilla having the general histological constitution of a taste bud.

As terrestrial life evolved, the taste buds disappeared from the outer skin and became concentrated in the mouth, and finally in the posterior part of the tongue (man).

1. Structure of the Taste Bud

Throughout the entire vertebrate phylum, the taste buds present a characteristic histological feature, namely the fact that all cells constituting the organ occupy its entire length, reaching from the apical free border to the base, in direct contact with the basal membrane. This is in striking contrast with the structure of sense organs belonging to the special somatic system (lateral line system, labyrinthine nerve endings) where the sensory cells occupy only the upper half or third of the entire height of the organ, their base never reaching the basal membrane. In bony fishes where both neurogems and neuromasts exist in the body skin, this feature permits an easy discrimination between both endings.

Classical descriptions of the taste buds are based mainly on the structure present in mammalian tongue (papilla foliata of the rabbit). It is generally accepted, and stated in many textbooks, that two kinds of cells are to be found in the gustatory end organ, sensory cells and supporting cells. The sensory cells are slender narrow elements with an elongated nucleus, a dark-staining cytoplasm tapering at its apical end and bearing a small cuticular conelike protrusion, the gustatory hair. The supporting cells are broader, often crescent-shaped, having a vesicular pale-staining nucleus. In addition to these two cell types, enveloping cells are described, the cornified ends of which form at the surface of the epithelium a small vesicular cavity (gustatory pore or gustatory pit) into which the gustatory hairs protrude (Fig. 10).

However, several eminent histologists (Retzius, Kolmer) have insisted on the fact that it is by no means always easy to distinguish those two cell types and that transitory forms between the two exist: a continuous process of differentiation could take place in the taste buds, as the older

sensory cells regress with pycnotic degeneration of their nucleus, and as younger ones take their place. According to Kolmer, the sharp distinction between sensory cells and sustentacular cells is really an extrapolation of the observations made on other sensory endings, such as neuromasts, where two cell types undoubtedly are present. The continuous formation of gustatory cells in the taste buds can be put in relation with the well-known fact that, after section of the afferent nerve in the barbels of *Ameiurus* (May, 1925; Torrey, 1934, 1936; Olm-

FIG. 10. Taste bud on the tongue of the hedgehog *Erinaceus europaeus*. Gustatory pore. No clear distinction between sensory and supporting cells.

sted, 1920a,b), the taste buds degenerate and reappear after the regeneration of the nerve.

Figure 10 represents a taste bud on the grooves of the papilla vallata of the hedgehog with a well-formed gustatory pore, opening at the surface by a small canal. Although dark and pale nuclei are to be seen, it is difficult to make a clear distinction between two separate cell types. Moreover, no distinct hairlike formation can be observed.

Recently, Murray and Murray (1960) examining in light microscopy or in phase contrast the taste buds of rhesus and cynomolgus monkeys, were unable to make a clear distinction between two cell types and concluded that there exists only one cell type, with variations in the

appearance of the cytoplasm, which can be vacuolated, or in the density of the nucleus. Moreover, no true "gustatory hair" extending into the pore could be seen.

The taste buds of bony fishes, located in the skin or on perioral barbels, are very similar to the gustatory endings of mammals. In *Ameiurus* their total number can be estimated as more than 100,000. They are located on the surface of the integument, their superficial end

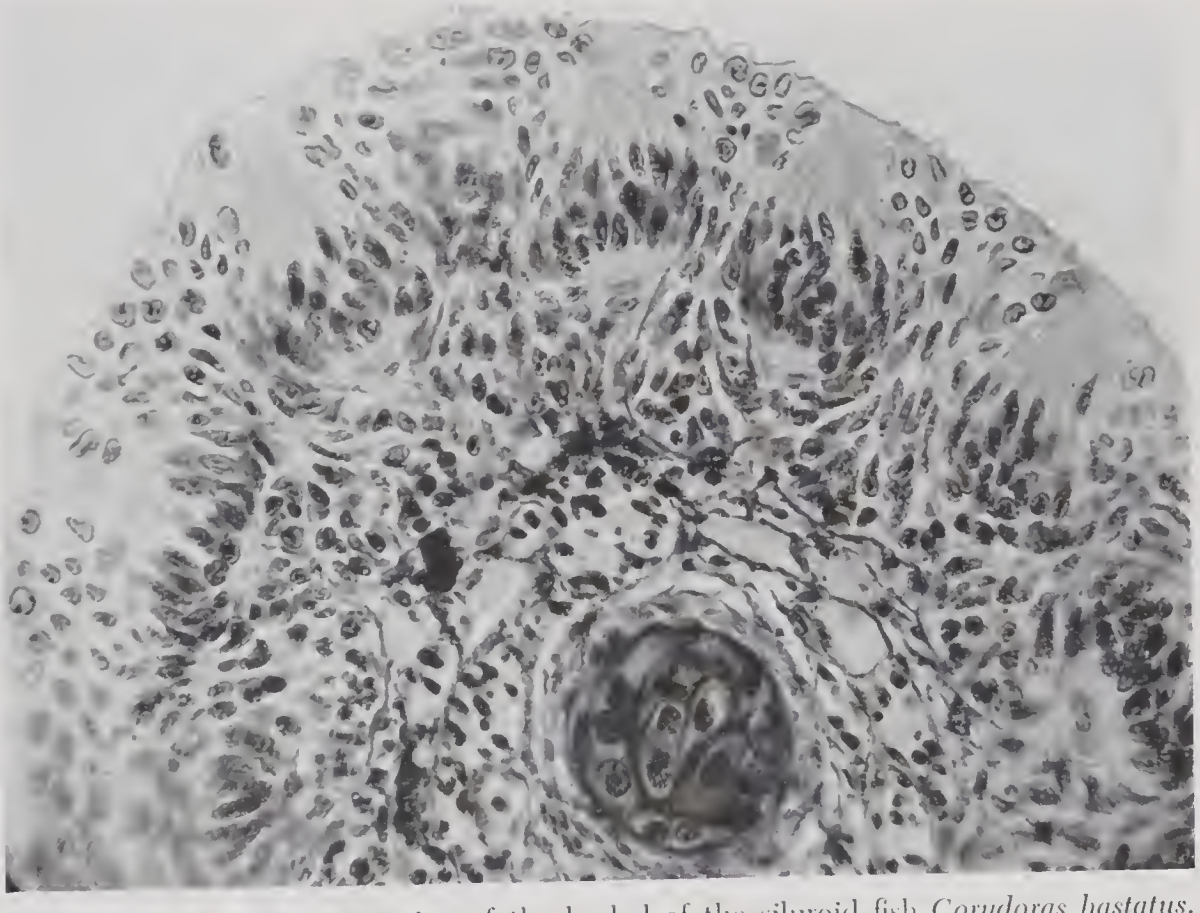

FIG. 11. Transverse section of the barbel of the siluroid fish *Corydoras hastatus*. Numerous taste buds (neurogems) in the epidermis.

being either level with the surface of the skin or presenting a slight depression; however, no true gustatory pore exists (Fig. 11). The buds can occupy the entire height of the epidermal epithelium; very often their height is less than that of the epithelium, and in this case, their base is supported on an enlarged dermal papilla (Fig. 12). Nevertheless the component cells extend throughout the whole organ from its free end to the basal membrane.

The teleostean neurogem is formed by numerous very slender cells with elongated nuclei situated in the lower half of the organ, where they form a transverse band. While at the base of the bud exists a clear cytoplasm, the apical parts of the cells taper to the surface and stain

definitely darker at their distal end. At the extreme basal part of the bud can be seen a peculiar cell with a long nucleus oriented transversally and a cytoplasm quite distinct from that of the gustatory cells themselves. Observations with light microscopy on the fish taste buds do not permit a clear distinction between sensory cells and supporting elements. The basal cell, however, is undoubtedly a distinct component of the bud.

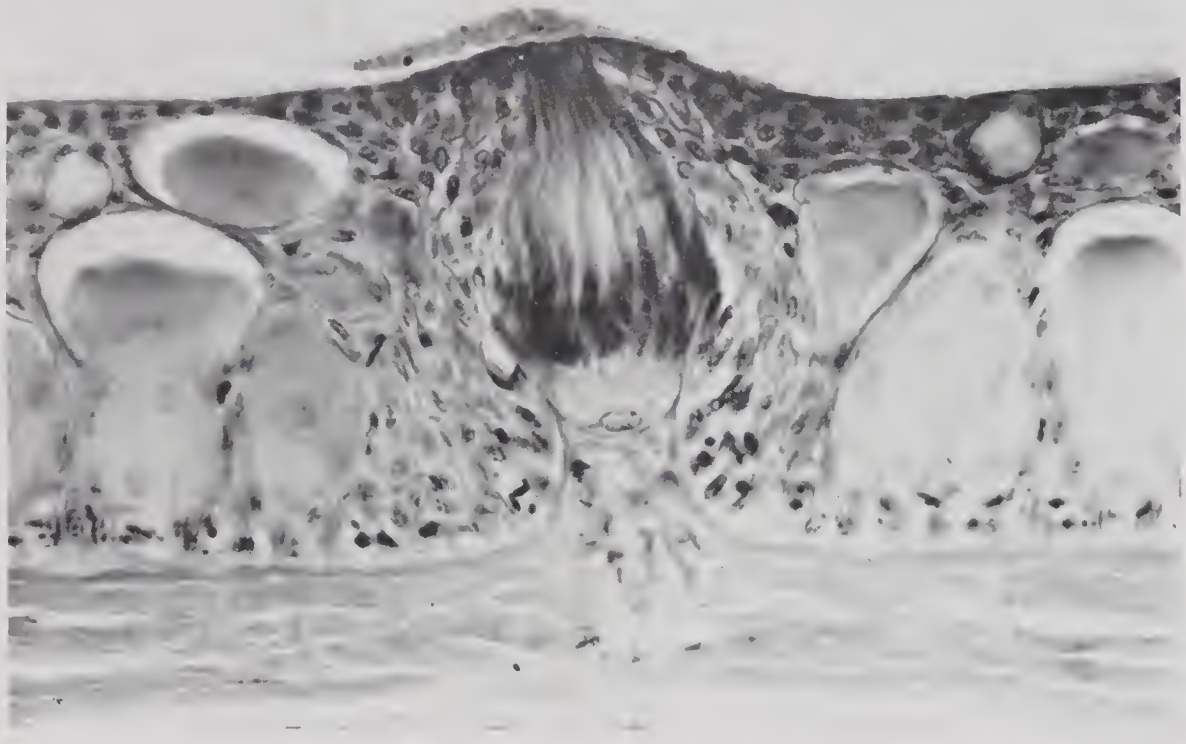

FIG. 12. A taste bud in the skin of the siluroid fish *Macronus*. Supporting and sensory cells cannot be distinguished. Note the basal cell.

It appears that investigations on the cytological structure of the vertebrate taste buds with light microscopy are unable to resolve the controversial question whether there are one or two cell types. Moreover, the presence of an apical hair protruding at the surface of the cells cannot be considered a constant cytological feature.

Electron microscope observations of the fine structure of the taste bud would therefore be expected to bring a definite answer to the conflicting opinions.

Such observations have been made by several authors on the taste buds of mammals, but their conclusions are not uniform.

In the taste buds of the rabbit de Lorenzo (1958) identifies two cell

types, sensory and supporting. The sensory cell contains, in addition to normal organelles, abundant vesicles throughout the length of the cell, and, at the apical pole numerous microvilli protrude into the gustatory pore. Sustentacular cells have a large clear nucleus and contain fewer granular or vesicular components; their apical regions do not reach the pore. No basal bodies nor true cilia exist at the apical pole of the bud. Engström and Rytzner (1956) and Trujillo-Cenoz (1957), on the other hand, are much more restrained in their description and do not make a clear discrimination between two cell types. Likewise, Murray and Murray (1960) in monkeys, deny the existence of distinct sensory and supporting cells; they describe "gustatory cells" in general, with terminal bars, small microvilli, and a dense material issuing from the tips of the cells and more or less filling the gustatory pit. Moreover, they observed the presence of concentric bodies, formed by multiple layers of densely packed membranes around a clear center (cf. Fig. 14).

In the siluroid fish *Corydoras*, the present author compared the structure of the taste buds on the barbels as seen in light microscopy and electron microscopy. Transverse sections of the bud (Fig. 13) are very instructive. They show indeed the presence of two definitely distinct cell types in the bud: (1) Cells with a regular circular contour contain mitochondria and numerous vesicles, generally located near the periphery; on longitudinal sections, the apical ends of these cells terminate with a small number of microvilli. (2) Cells with very irregular contours form a mosaic-like pattern around cells of the first type. They contain fewer vesicles but present a very conspicuous Golgi apparatus and a few ergastoplasmic sacs. Occasionally desmosomes can be seen at the limit of some cells. The two cell types forming the bud can be reasonably interpreted as sensory cells and supporting cells. They are totally different from the surrounding epithelial cells, being characterized by their long nuclei with axis parallel to the skin surface and by their content of typical tonofibrils; numerous desmosomes are to be seen at many points of the contiguous cell membranes. When sections passing through the nuclear regions of the buds are examined, a very peculiar aspect of the nuclei is to be seen. The outer limit of the nuclei is scalloped and shows multiple cytoplasmic invaginations (Fig. 14).

It has been impossible to state as yet with precision whether these nuclei belong to sensory or to supporting cells, or if there are any differences between the nuclei of the cell types.

Taste buds are richly innervated and numerous fine amyelinated fibers have been demonstrated between the cells, running nearly to the upper surface. It is well known that the epithelial regions between the

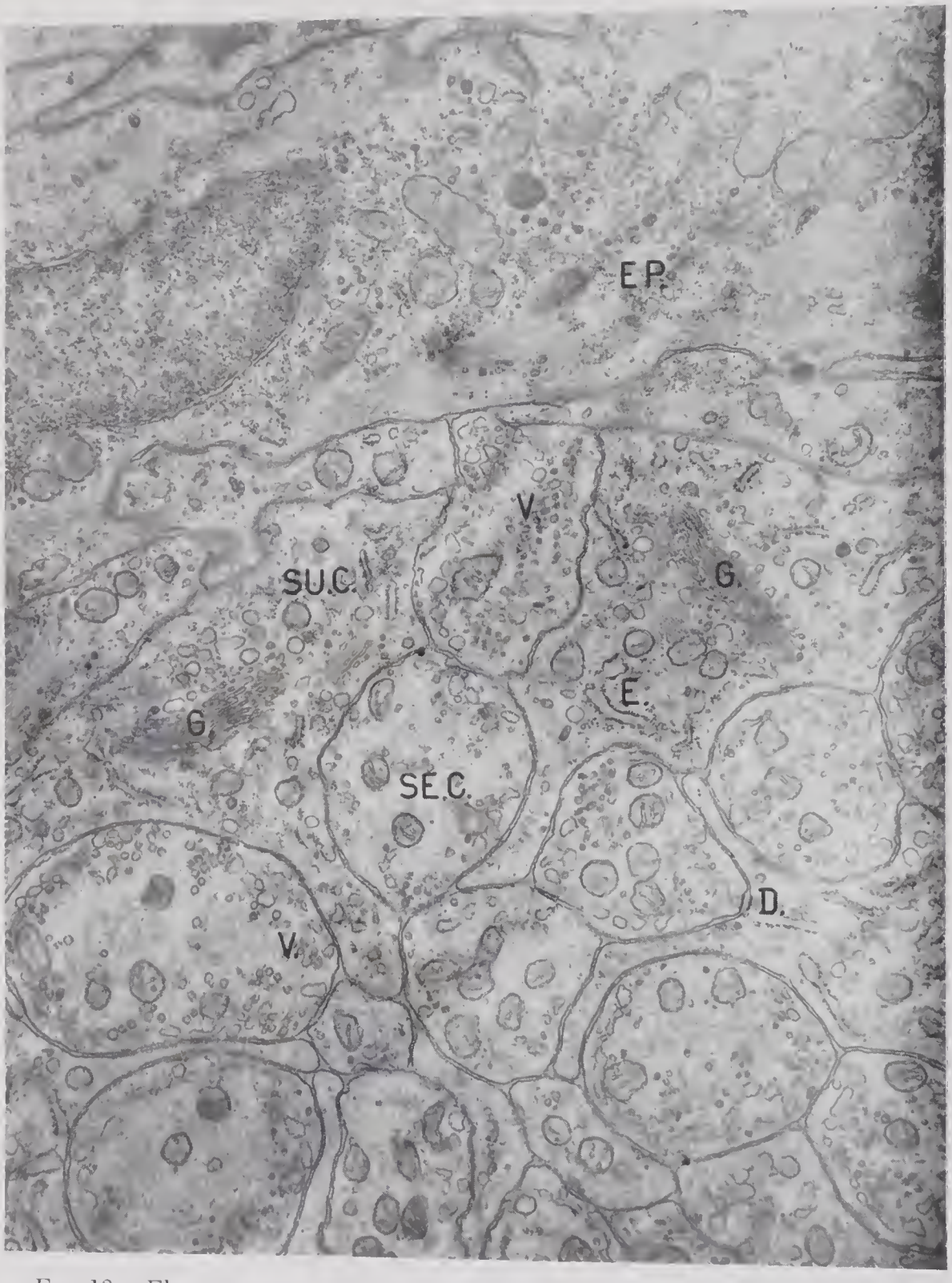

FIG. 13. Electron microscope photograph of a transverse section through a taste bud of *Corydoras* (upper region). Two cell types are easily distinguished: sensory cells (*SE.C.*) circular in contour and containing multiple vesicles (*V*) and a few mitochondria; supporting cells (*SU.C.*), with mosaic-like contour, containing ergastoplasm and ribosomes (*E.*) and Golgi material (*G.*) *EP.*: epidermal cell with tonofibrils. EM Siemens. Magnification: × 14,625. From Cordier and de Harven (unpublished data).

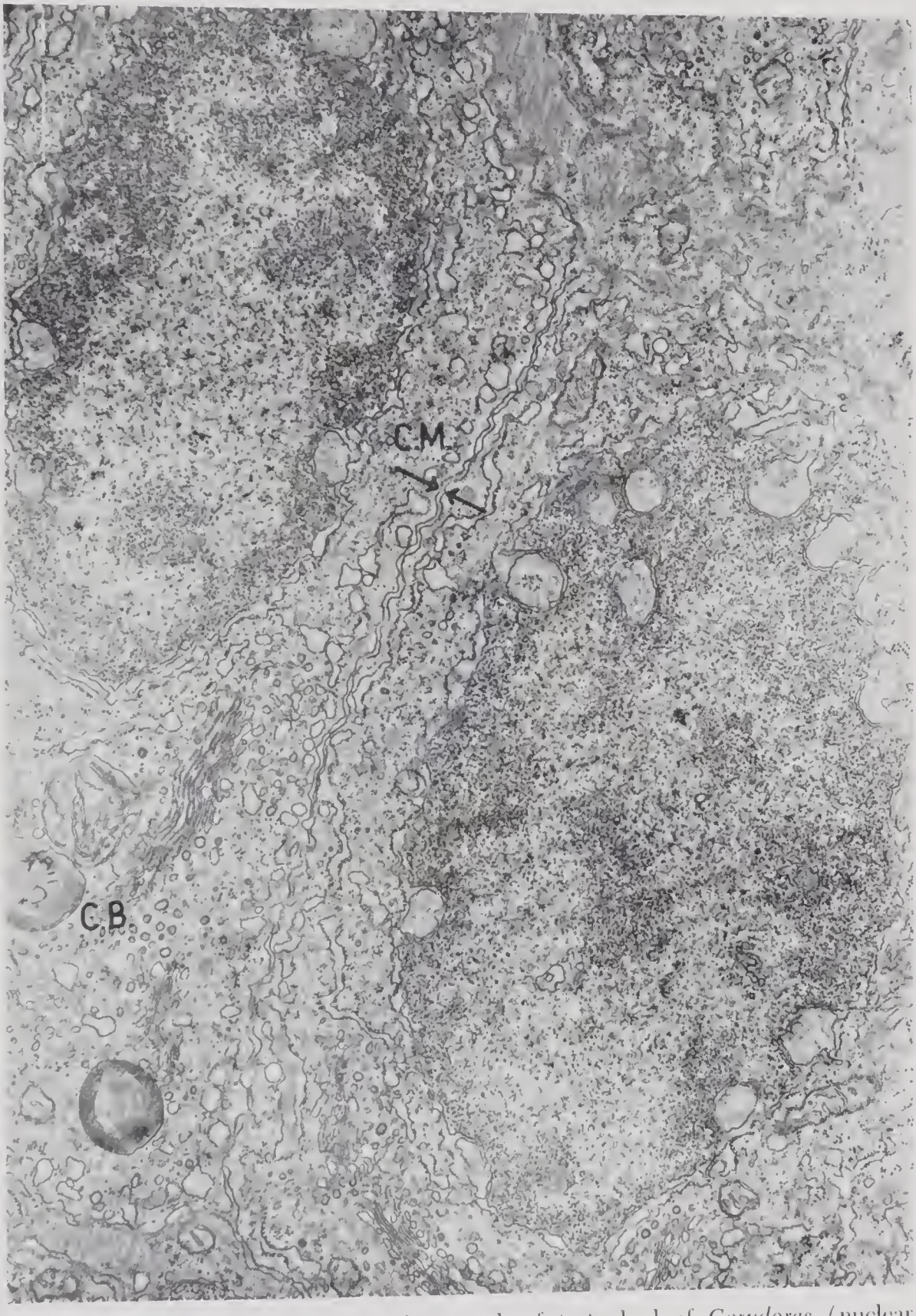

FIG. 14. Electron microscope photograph of taste bud of *Corydoras* (nuclear region). Intranuclear cytoplasmic invaginations. Concentric multilayered bodies are to be seen at lower left. EM Siemens. Magnification: × 14,700. From Cordier and de Harven (unpublished data).

buds are equally richly innervated and that a same fiber can distribute its end ramifications both through a taste bud and into the surrounding epithelium (intra- and extragemmal fibers). This disposition has led to the suggestion that the whole territory innervated by glossopharyngeal fibers could be gustatory.

The observations of de Lorenzo (1958) with the electron microscope have shown that the sustentacular cells form sheaths around the ascending naked nerve fibers, their membranes forming a kind of mesaxon around them. In the observations on the siluroid fish, the nerve fibers seem to be limited to a subnuclear region; however, occasionally a very small fiber is to be seen ensheathed completely by the membrane of a sustentacular cell.

2. Histochemical Data

Baradi and Bourne (1953) have studied the localization of several dephosphorylating enzymes in the taste buds of the rabbit and other mammals (monkey, bat). They conclude that the epithelium which lines the gutters of lingual papillae appears to contain several such enzymes (acid and alkaline phosphatase, adenosine triphosphatase, β-glycerophosphatase). Some of these are located at the base of the bud, others at the apex. The cells themselves are poorer in enzyme content than the surrounding epidermal cells. Various tasting substances affect the activity of the different enzymes in different sites. These facts suggest a possible functional interpretation of gustatory sensitivity. Multiple substances of different sapidity could affect the activity of the different enzymes present in the buds, either inhibiting them or reinforcing their action, thus creating changed ionic conditions which could be the origin of the nerve impulse. However, as in so many other sense organs, neither morphological nor histochemical research has yet brought definite information on the mechanism of the gustatory discriminating function.

B. Sensory Cells for Special Somatic Sensitivity

1. The Lateral-Line System

In fishes, in larval Amphibia, and in some adult Amphibia, a very complex sensory system exists, very polymorphic from the anatomical point of view, and generally known as the lateral-line system. This term alludes to the existence in many fishes of a very conspicuous lateral line of sense organs on both sides of the trunk. However, the head region is largely supplied with sensory organs of the same system; therefore the term "lateral system" is really very restrictive and must be accepted in

a larger sense to designate the whole array of nerve endings belonging to this extensive group.

As to its function, the lateral-line system is generally considered as being able to perform delicate detection of water movement, although other modalities of receptivity have been discovered in some of them (e.g., sensitivity to variation of temperature or to electrical stimulation). Its organs are innervated by the two nervi laterales, anterior and posterior. However, the nerve fibers, in order to reach the terminal organs, follow the course of other cranial nerves, namely the branches of the VIIth and perhaps the Vth, nerve (the latter course being controversial), for the head sensory organs and the branches of the Xth nerve for the trunk organs. In the oblongata are special centers, containing secondary sensory neurons of the second order; these centers are known as lobi laterales anterior and posterior.

The lateral-line organs present a great variety of anatomical aspects, and comparative anatomy has found an extensive and interesting field in their description and identification.

In making a general survey of the great variety of anatomical patterns of the organs of the lateral system, it seems appropriate to arrange them in a progressive series according to the remoteness of the sensory regions from a free epithelial surface. This results in the following classification.

1. Superficial "neuromasts" located in an epithelial layer, and level with the surface of the latter, either on the skin surface or in the epithelial lining of lateral intradermal canals.

2. Sensory endings located in a more or less deep invagination of the skin (pit organs) or at the bottom of glandlike short tubules opening freely onto the surface: organs of Fahrenholz, mormyromasts.

3. Sensory vesicles situated deep in the dermis and in open communication with the surface by long flexuous canals: ampullae of Lorenzini.

4. Closed sensory vesicles having no connection at all with the surface: vesicles of Savi.

If now the histological and cytological aspects of the lateral-line sense organs are examined, it appears that in the majority of them we find a typical structure having the general characteristics of sensory epithelium for special somatic sensitivity and closely similar to organs of the sensory regions of the labyrinth (maculae, cristae, auditory regions). We will term these structures typical lateral sensory epithelia.

However, in some organs of this group, the cytological structure

deviates from this typical pattern and the aspect and arrangement of the cells present peculiar features; we will describe them as atypical lateral sensory epithelia. It can be stated, moreover, that for several such organs with atypical sensory epithelium, experimental research has demonstrated a function distinct from the general role attributed to the lateral-line system, i.e., detection of mechanical stimuli arising from water movements and from the direct contact of the animal with its watery environment.

The structure of the lateral-line organs will now briefly be described according to the proposed classification.

a. The typical lateral sensory epithelia. In this group the sensory endings are formed by two very distinct cell types, sensory cells and supporting cells.

The *sensory cells* are pear-shaped elements, with a tapering apical pole, terminating with a dense cuticular structure from which arises a sensory hair protruding at the free surface. The most characteristic aspect is the fact that the sensory cells occupy the upper half or third of the total height of the epithelium and that their base never reaches the basal limit. The terminal hairs are very often in close contact with a jellylike amorphous membrane covering the sensory ending, which may be totally lacking, especially in superficial sensory epithelia. The sensory cells present generally a fibrillar cytoplasm. In some cases they contain large globular inclusions in the immediate vicinity of the nucleus; in *Protopterus* (Fig. 15) and *Polypterus* (Fig. 16) these inclusions stain deeply with iron hematoxylin and before staining they present a very distinct yellowish color. A chromolipoid nature seems probable; the inclusions are easily to be found in young animals, but they seem to disappear nearly totally in adult specimens. In some teleosts (Fig. 17, *Xiphophorus*) similar formations are to be seen, but they appear rather as large round vacuoles or globules, which contrast, by their unstained aspect, with the deeply staining cytoplasm.

The *supporting cells* are cylindrical elements with basally located nuclei. Their apical poles form a superficial limiting membrane, which on tangential sections forms a mosaic-like pattern through which at regular distances appear the apical extremities of the sensory cells. Distinct terminal bars mark the boundaries of the supporting cells. Very often tonofibrils are to be seen in their cytoplasm.

A typical sensory epithelium of this type is to be found, as stated, in the following organs.

i. Neuromasts. A neuromast is a barrel-shaped organ or bud, located in the epithelium and not very dissimilar in appearance from a taste

bud or neurogem. However the characteristic position of the sensory cells eliminates all possibility of confusion between neuromasts and neurogems, specially when both types of sensory ogans are round in the superficial skin, as in many fishes. Neuromasts exist on the outer skin of many teleosts and larval amphibians (Fig. 17). In adult *Xenopus* the small whitish lines easily observed with the naked eye, on the skin of head or body, contain numerous neuromasts of this type.

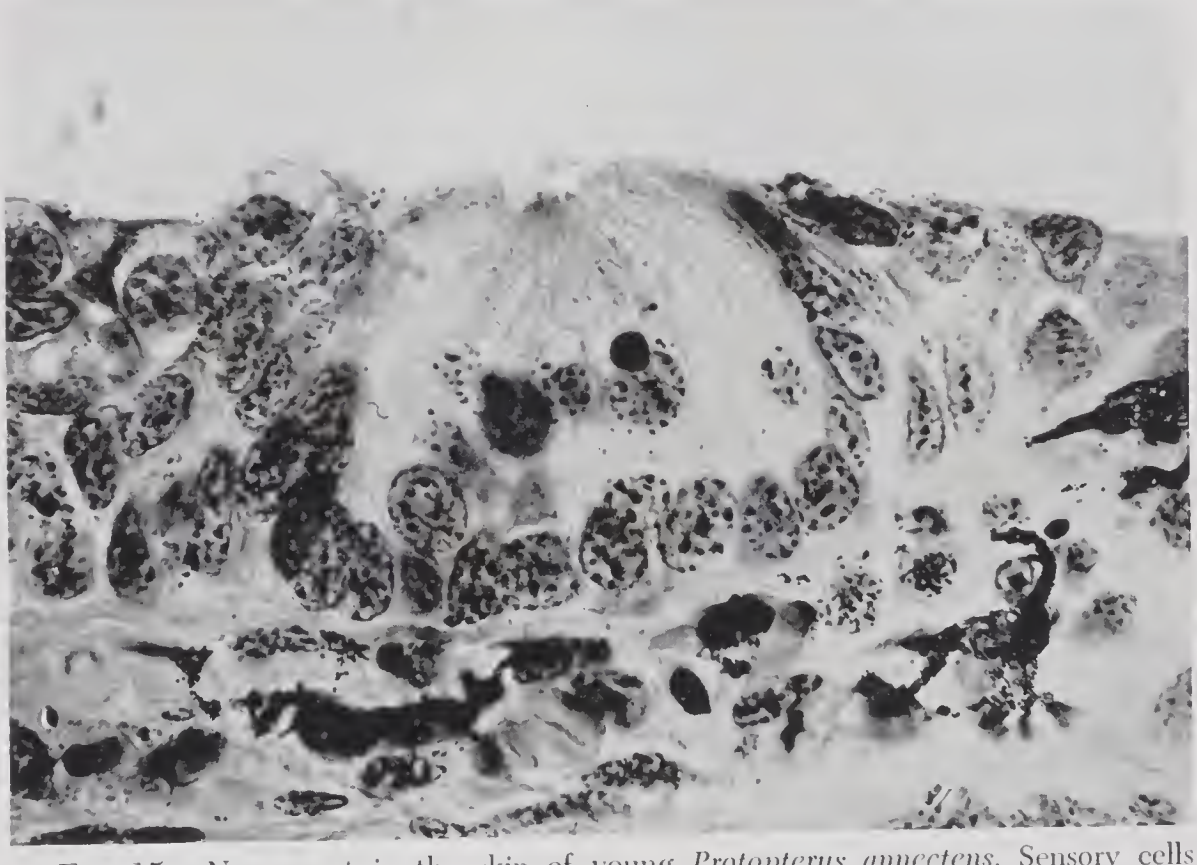

FIG. 15. Neuromast in the skin of young *Protopterus annectens*. Sensory cells with apical dense extremities and supranuclear inclusions. A small "cupula" is visible. Supporting cells with basal nuclei.

Neuromasts can also be encountered in the epithelial lining of the lateral canals. Typically, there exists in most fishes a deep intradermal canal on each side of the body, opening at regular intervals at the surface of the skin by little pores. On the head, three identical canals are described in supraorbital, infraorbital, and maxillary positions. The precise course of these lateral canals, the exact location of their pores and of the intracanalicular neuromasts have been very accurately studied in many species [see, e.g., Herrick (1903) in *Ameiurus*].

The intracanalicular neuromasts present the same general structure as superficial neuromasts; however, the covering cupula is often better preserved and more conspicuous (Fig. 18).

In some cases, the intracanalicular ending enlarges and expands into a broad thickened sensory area, which, in accordance with the aspects encountered in the sacculus and the utricle of the labyrinth, must be called a macula (macula lateralis) (Gérard, 1936). The term "neuromast"

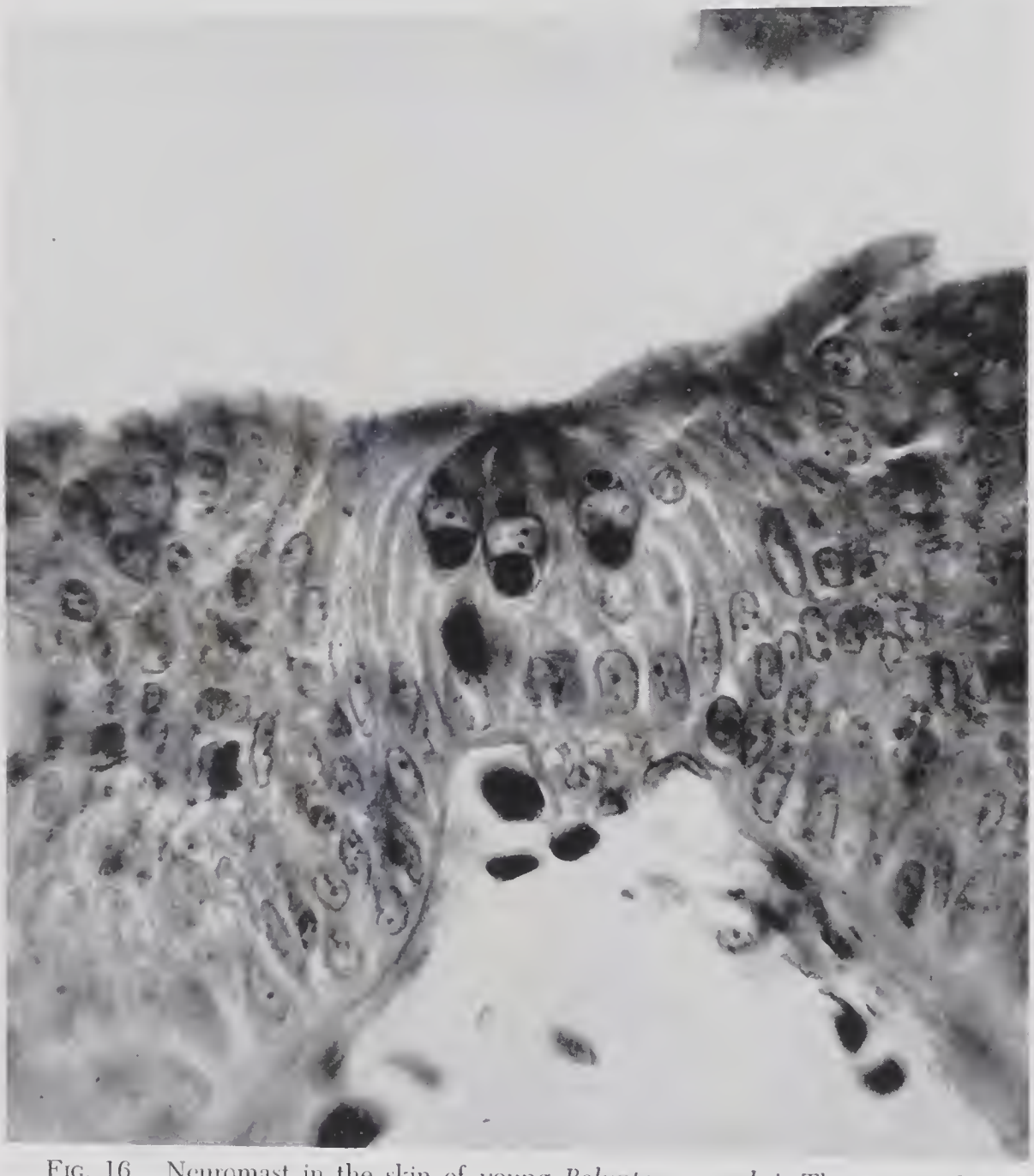

FIG. 16. Neuromast in the skin of young *Polypterus weeksi*. Three pear-shaped sensory cells with infra- and supranuclear pigmented inclusions.

is not justified in this case, since it must be reserved for lateral-line endings appearing as barrel-shaped buds. In *Polypterus*, a further interesting feature has been observed on these maculae laterales, namely the presence of a true otoconial membrane, loaded with numerous calcium-

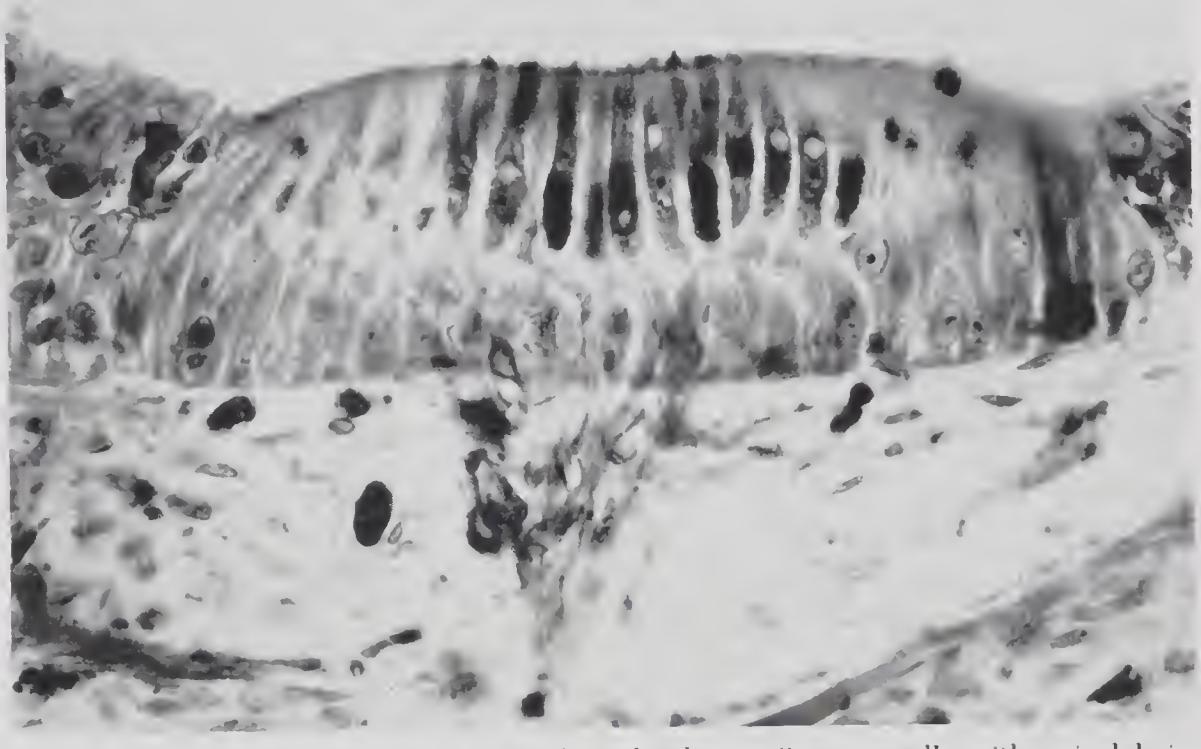

Fig. 17. Neuromast in the skin of *Xiphophorus*. Sensory cells with apical hairs and supranuclear unstained globules.

Fig. 18. Neuromast in a lateral canal of *Scyllium*. Sensory cells with apical thickenings.

containing corpuscles. The histological and cytological characteristics of these lateral-line endings are thus identical with those of true labyrinthine maculae (Fig. 19).

ii. Organs of Fahrenholz. Fahrenholz (1927) has described in the outer skin of the lungfish *Lepidosiren* tubular glandlike structures provided at their deeper part with sensory cells which, on the basis of their characteristic appearance, belong to the lateral-line system. Similar organs have been found in *Protopterus* (Cordier, 1936) and in *Polypterus.*

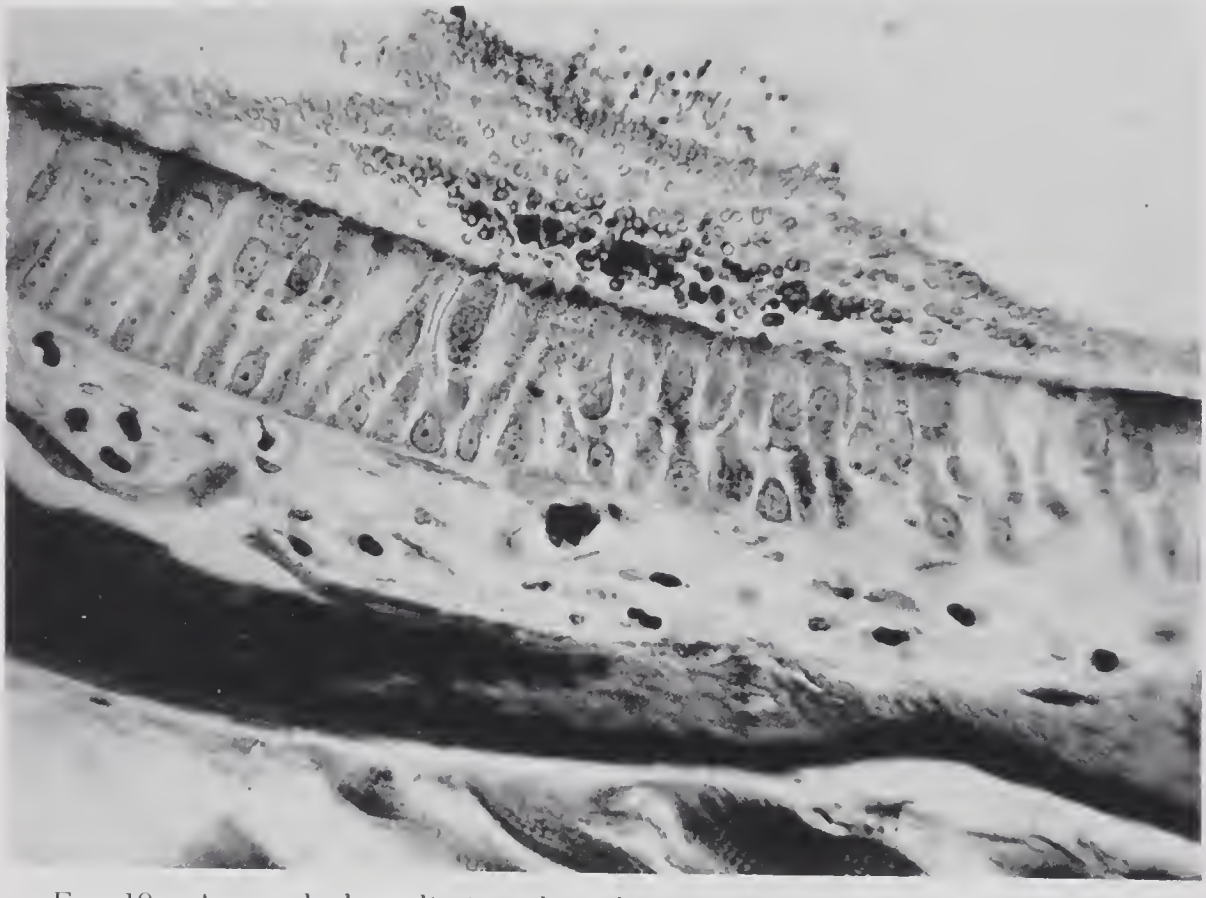

FIG. 19. A macula lateralis in a lateral canal of *Polypterus weeksi.* Numerous piriform sensory cells. An "otoconial" membrane with abundant concretions.

Figure 20 represents a typical organ of this kind in *Polypterus;* the characteristic pear-shaped sensory cells are easily seen, and the structure of the bottom of the crypt is exactly that of a typical lateral sensory ending. In *Protopterus,* Cordier could observe the presence of one or two yellowish inclusions identical with those found in the skin neuromast. Moreover, in the lumen of the tubule, a spheroidal mass exists, formed of concentric layers and suggesting a close resemblance with otoliths of fishes; preserved with Bouin-Hollande fluid, which contains 2.5% copper acetate, these concentric masses seem to be encrusted with copper salts, exactly as in the case of bone or teeth. The organs of

Fahrenholz seem to be well developed only in young specimens and to shrink in the adult, with disappearance of the typical sensory cells.

iii. Vesicles of Savi. In the rostrum of the electric skate *Torpedo*, very peculiar sense organs were described in 1844 and are known as vesicles of Savi (Savi, 1844). They appear as small (2–3 mm. in diam-

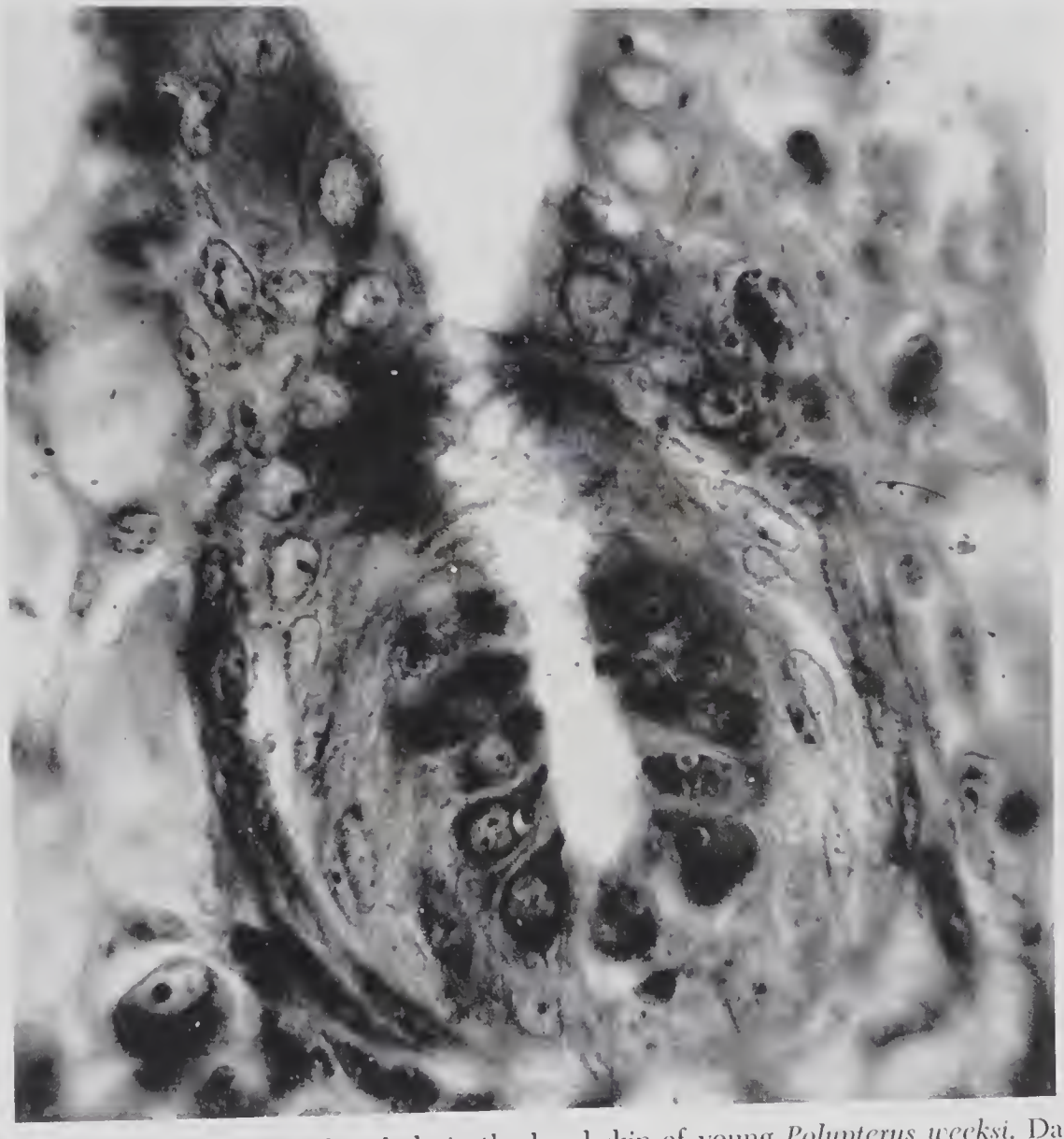

Fig. 20. Organ of Fahrenholz in the head skin of young *Polypterus weeksi*. Dark piriform sensory cells with pointed apical extremities.

eter), closed, cystlike formations situated in linear groups in the dermis, rostrally to the opening of the nasal cavity. They are embedded in a very loose gelatinous interstitial tissue and are attached to a tendinous or chondroid band.

On transverse section, each vesicle appears as a domelike formation, adhering with a flattened base to the connective band. The vesicle is

lined with a flattened epithelium, which in the basal regions forms three distinct circular thickenings, the middle one being a bit broader than the two lateral ones. These disks present the characteristic histological structure of sensory endings belonging to the special somatic system. They contain pear-shaped sensory cells, with an apical hair, which are located in the upper half or third of the epithelium and are surrounded by supporting cells with basal nuclei (Fig. 21). Each disk

FIG. 21. Four vesicles of Savi in the head of *Torpedo marmorata*, placed in a row on a chondroid band. In one of them the three sensory areas are seen with afferent nerve fibers. "Cupulae" cover the sensory areas.

is covered by a gelatinous amorphous membrane and innervated by thick nerve fibers which perforate the connective band.

The structure of the epithelial disks of the vesicles of Savi indicates that they belong to the lateral-line system. No information on their possible function is at present available. It has been suggested that they could be mechanoreceptors, receptive to mechanical pressure transmitted by means of the semifluid surrounding tissue. This special sensitivity could be related to the fact that the electric skate lives in sandy sea bottom and digs itself into the sand by means of the rostrum. However, no experimental confirmation of any kind has been attempted, and the exact function of the vesicles remains entirely mysterious.

b. The atypical lateral sensory epithelia. In this type of sensory ending, the characteristic structure found in the previously described organs is modified, the essential fact being that the sensory cells are not located in the superior half of the epithelium and that they can present cytologic features deviating more or less sharply from the typical aspect.

Atypical sensory epithelium is formed (1) in the ampullae of Lorenzini, (2) in several types of sensory organs of the Mormyridae (teleostean fishes).

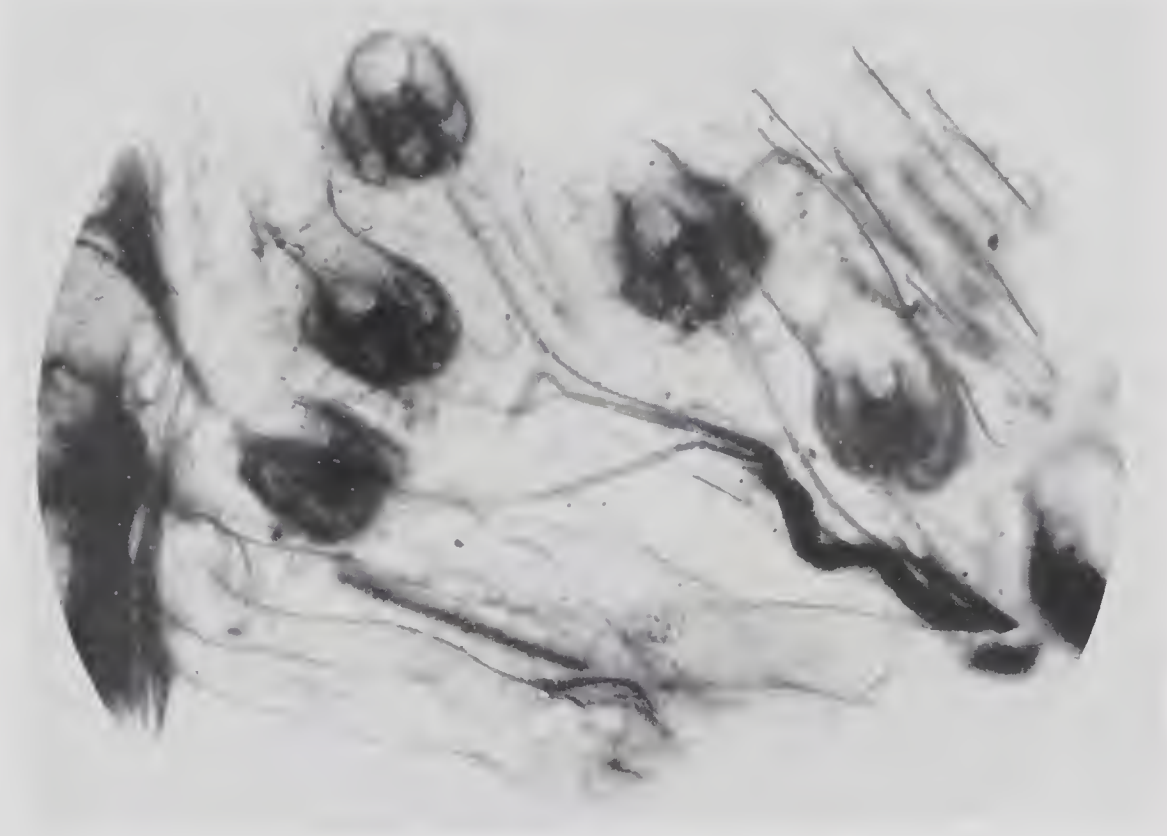

Fig. 22. Ampullae of Lorenzini of *Scyllium*. Total preparation showing a few ampullae with afferent nerves and, in the lower part of the picture, the connecting canals.

i. The ampullae of Lorenzini. The cephalic region of Selachii, both sharks and skates, contains peculiar sensory organs located in the dermis and totally distinct from the lateral-line organs. They are known as ampullae of Lorenzini and were described for the first time in *Torpedo* in 1678 (Lorenzini).

The ampullae of Lorenzini are groups of vesicles which are in open communication with the skin surface by means of long flexuous canals filled with a gelatinous substance and opening by superficial pores (Fig. 22). The ampullae are the terminal dilatations of these canals and present a small number of peripheral diverticula, circularly disposed

around the axis. At the point where a diverticulum opens into the lumen of the ampulla, a central flattened protrusion exists, forming the anvil-shaped central plate (*Zentralplatte*, Dotterweich, 1932) (Fig. 23). In some species of Selachii, the morphology can be more or less complicated, the diverticula being spheroidal appendages or elongated tubules.

Typical ampullae of Lorenzini have also been discovered in one species of teleost, the siluroid fish *Plotosus anguillaris* (Friedrich-Freeksa,

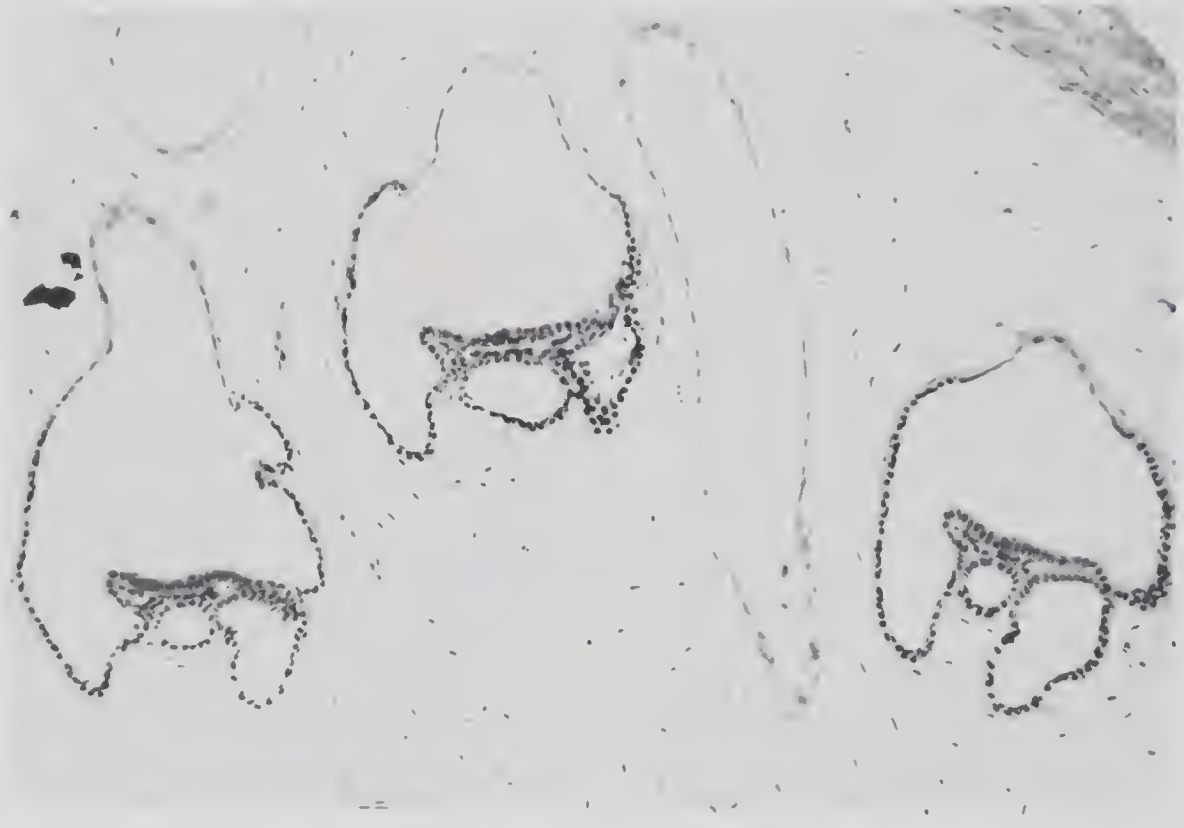

FIG. 23. Three ampullae of Lorenzini of *Scyllium* with the central plate and the peripheral diverticula.

1930). However, the ampullae do not present diverticula and are simple spheroidal dilatations of the terminal end of the canals.

In the very loose connective tissue surrounding the ampullae, numerous myelinated nerve fibers are seen.

At the base of each ampulla (Kantner *et al.*, 1962), the nerve bundles expand fanlike, their terminal branches following a recurrent course, the entire disposition being reminiscent of a weeping-willow tree. After losing their myelin sheath, the fine neurites form a subepithelial network, articulating finally with the base of the sensory elements.

The cytological structure of the Lorenzini epithelium has been described by Retzius (1898) and Dotterweich (1932) in Selachii and

by Friedrich-Frecksa (1930) and Gérard (1947) in *Plotosus*. The whole inner surface of the ampullae in Selachii, with exception of the central plate, is lined with an epithelial layer formed by two distinct cell types. The statement of Herrick (1903), according to which the histological structure of the ampullae of Lorenzini corresponds to that of the neuromasts, cannot be retained. Indeed the common cytological pattern so characteristic of typical sensory epithelium of the lateral system and of the labyrinthine nerve endings is not to be found in the ampullae.

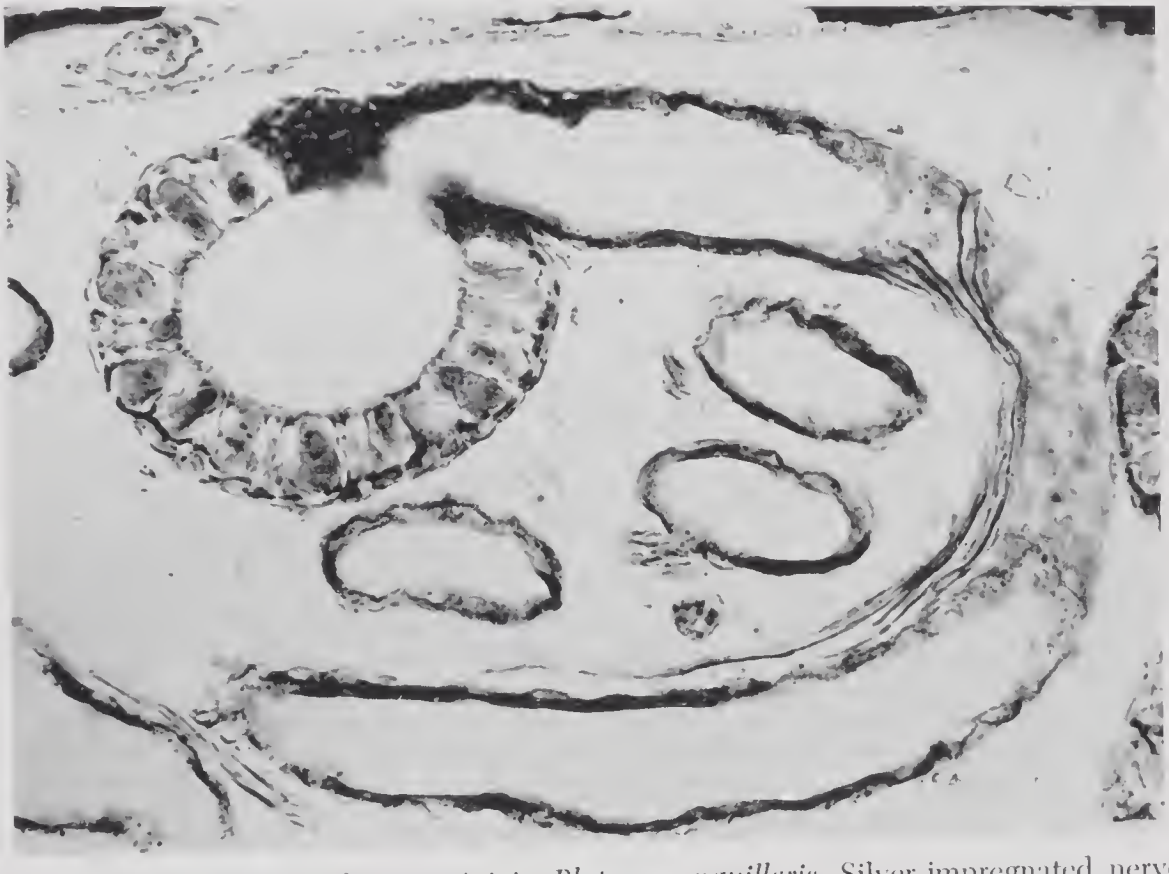

FIG. 24. Ampulla of Lorenzini in *Plotosus anguillaris*. Silver-impregnated nerve fibers are seen running parallel to the connecting canal and at the base of the sensory epithelium of the ampulla.

The two cell types met with in the ampullar epithelium are: (1) large ovoid cells, with central nucleus and with narrowing apical pole on which no sensory hair can be seen; (2) intercalated cells shaped like an inverted pyramid with the large base directed upward and forming the main part of the epithelial surface while the other end is directed basally and reaches the basal membrane with its tapering extremity. The nucleus has a triangular shape, stains deeply, and is situated in the upper large portion of the cell.

In the teleost *Plotosus anguillaris*, a very similar though not quite identical cytological structure is observed in the terminal ampullae.

Large ovoid cells with tapering apical poles alternate with intercalate cells, having generally basally located nuelei; their apical poles enlarge and form a limiting membrane through which the end of the ovoid eell protrudes at a regular distance (Figs. 24 and 25). Tonofibrils are easily to be seen throughout the entire length of these cells.

The high degree of innervation of the ampullae and the thickness of the nerve fibers that reach them indicate their sensory nature. Whieh of the two kinds of cells is the sensory element? Dotterweieh (1932) eon-

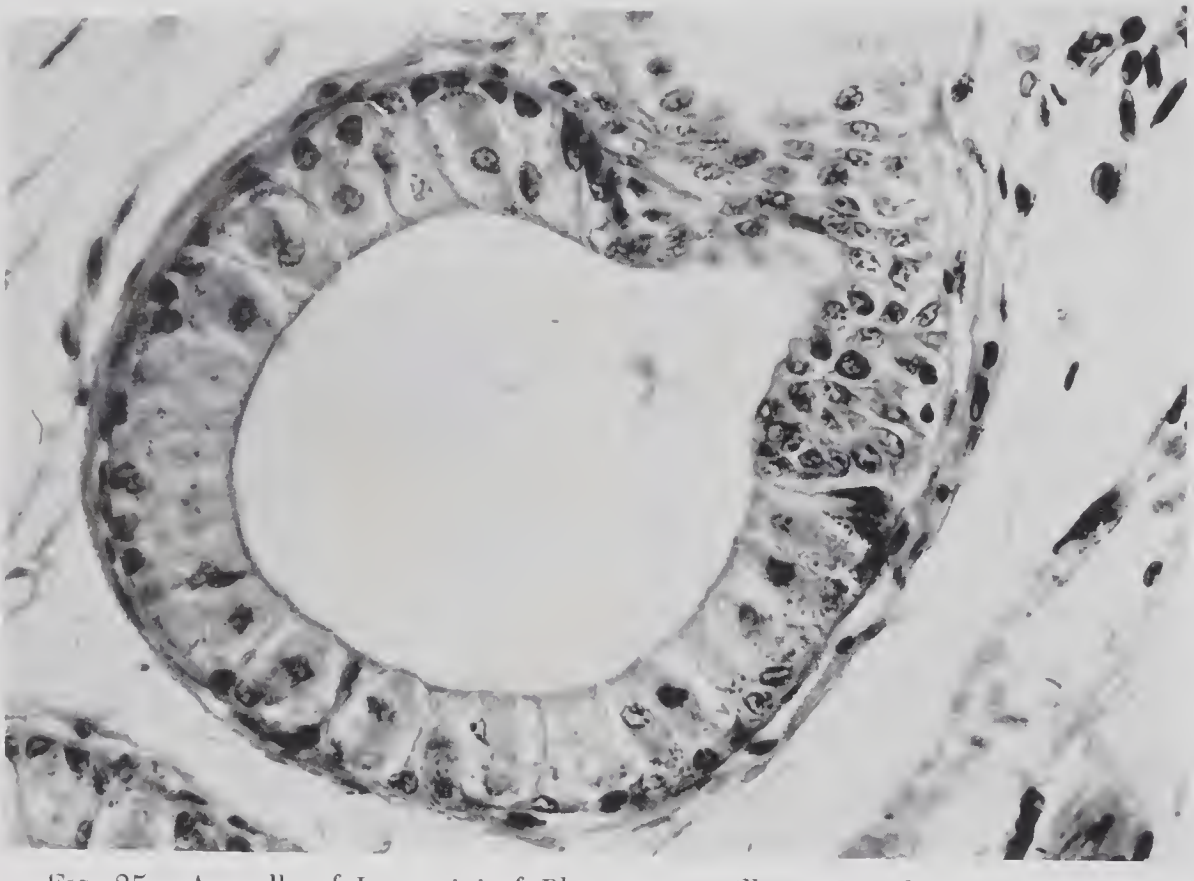

FIG. 25. Ampulla of Lorenzini of *Plotosus anguillaris*. Ovoid sensory cells alternate with narrow supporting cells.

sidered the large ovoid cells to be glandular elements secreting the gelatinous content of the canal; the pyramidal cells were interpreted as primary sensory cells, their tapering lower end being eontinuous with a nerve fiber.

Retzius (1898) was of the opposite opinion and believed the large ovoid cells to be sensory elements. P. Gérard, in *Plotosus,* observed, after silver staining, the formation of synaptie eonnections at the base of the ovoid cells, this relation appearing very elearly on tangential or oblique sections. The ovoid eell should thus be eonsidered as the sensory elements. Kantner *et al.* (1962), investigating on silver-impregnated sections the

innervation of the ampullae, were unable to make a distinction between the two cell types.

The evidence seems thus to be conflicting. However, the cytological features of the epithelium of Lorenzini and the comparison with other sensory epithelia points very strongly to the sensory nature of the ovoid cells, whereas the pyramidal cells are to be considered as supporting cells. The shape of the pyramidal cells in the ampullae corresponds

FIG. 25A. Electron microscope photograph at level of the basal synapse in an ovoid sensory cell of the ampulla of Lorenzini of Torpedo. Evagination of the cytoplasm of the sensory cell (cs) into the nerve fiber (n). The characteristic ribbon with numerous synaptic vesicles is seen in front of the synaptic junction. ca, supporting cell. From Barets and Szabo (1962).

exactly to the appearance of the supporting cells in the pineal organ (*Rana*, Kelly and Van de Kamer, 1960) or in the saccus vasculosus of fishes: a large superior extremity with a triangular nucleus, and a narrow tapering inferior extremity reaching the base of the epithelial layer.

A recent electron microscope investigation on the ampullae of Lorenzini of Torpedo (Barets and Szabo, 1962) has brought definite proof of the sensory nature of the ovoid cells and interesting information on

the structure of the synapse. At the base of the cells several cytoplasmic evaginations are found, protruding in gutter-shaped depressions of terminal nerve fibers. A presynaptic dense rod or ribbon, closely surrounded by synaptic vesicles, is constantly present in front of these evaginations (Fig. 25A). This remarkable structure strongly resembles the crescent-shaped plate in the terminal swelling of the retinal rod-cell at the level of the synaptic junction with the dendrites of the bipolar cell. Moreover, it is identical with the dense rod described by Smith and Sjöstrand (1961) at the base of the outer hair cells in the organ of Corti.

A further morphologic feature should be mentioned. In the ampullae of Selachii, the central anvil-shaped part is covered by an entirely different type of epithelium, in which the two characteristic cell types of the other regions are absent; the plate presents a cylindrical epithelium having clear cells without special cytological features. However, in young specimens the present author has found rare ciliated cells, occupying the upper part of the epithelium and reminiscent, by their position, of the characteristic elements of true neuromasts.

The function of the ampullae of Lorenzini of *Scyllium* has been experimentally investigated by Sand (1938), and by Hensel (1955, 1956, 1957). Both authors demonstrated the existence of a continuous rhythmic electrical activity in the afferent nerve when the environmental temperature remains constant. A very small drop in the temperature results in rapid increase of the frequency of the discharge, whereas a rise in temperature is followed by diminution, or by total inhibition, of discharge. The ampullae of Lorenzini are therefore considered as specific cold receptors. Kantner compares the anatomical disposition of the nervous net at the base of the epithelium with that described in the tongue of the cat. In both cases, sensitivity to cold is related to the presence of a very superficially located nervous network. However, it is possible that the high specific receptivity of the Lorenzini ampullae to cold should not be accepted as their only function. In a recent investigation Murray (1962) was able to obtain electrical responses of the afferent nerve, in *Raja,* by electric voltage differences in the environment. Furthermore the frequency of discharges increased when a fine stream of diluted saline water was directed in the direction of the opening of the ampullae and the inverse result was obtained with concentrated sea water. The results of Murray demonstrated the sensitivity of the ampullae of Lorenzini to electrical stimuli and to changes in the salt content of the water from 3 to 5%. Moreover the same author, contrary to the results of Hensel, was able to elicit responses by means of mechanical stimuli.

The ampullae of Lorenzini furnish thus an excellent example of a

sensory organ in which the concept of specificity undergoes a definite defeat. As thermoreceptors, they can be put in parallel with two other thermoreceptors, the function of which has been closely investigated by physiological means, i.e., the pit organ of pit vipers, and the cold and warm receptors of the mammalian tongue. In the ampullae a well-differentiated epithelial component is present: in the tongue, no specific sensory cells exist, whereas in the pit organ of snakes (see below) the presence of "sensory cells" is still a matter of discussion. However, a common histological feature exists in each of those three thermoreceptor endings, i.e., a nervous network very superficially located (cf. Kantner *et al.*, 1962).

Finally, if we consider the problem of the ampullae of Lorenzini from a purely biological viewpoint, it is difficult to discern in what degree the animal could benefit from its ability to perceive a minute drop of temperature in the environing sea water, and what importance such a discriminative sensitivity could have for its behavior.

ii. The lateral-line organs of Mormyridae. The African fishes of the family Mormyridae comprise a zoological group of very great interest from a biological and a neurological point of view. The brain of these fishes is remarkable by the monstrous development of the valvula cerebelli ("mormyro-cerebellum"), which sprouts mushroom-like between the two halves of the mesencephalon and, extending forward and backwards, covers the entire dorsal aspect of the encephalon. Furthermore, the lateral regions of the oblongata (region IV of Herrick) are hypertrophied and, being confluent on the midline, cover the roof of the IVth ventricle. To this extraordinary hypertrophy of all centers in relation with the lateral-line system, corresponds the existence of remarkable sensory endings in the skin, innervated by the thickly myelinated fibers of the lateral nerves. Three types of endings will be mentioned: (a) the so-called "*Schmauzenorgane*" (Stendell, 1916), which for their unique and exceptional histological features could deserve the denomination "mormyromasts" (Cordier, 1937); (b) the so-called "*Knollenorgane*" (Stendell, 1916; Gérard, 1940), or bulbous organs of the skin; (c) the maculae laterales of the lateral canals of the head.

(a) The mormyromasts. The peculiar sensory endings called "mormyromasts" are found in great number in the skin of the snout of the Mormyridae, in lesser number on the head; even on the body skin some of them are to be found. The most curious feature of these organs is the fact that their histological structure cannot be considered as a fixed one; on the contrary, the organs are very plastic and, even in the adult, are the seat of continuous transformations. The different aspects

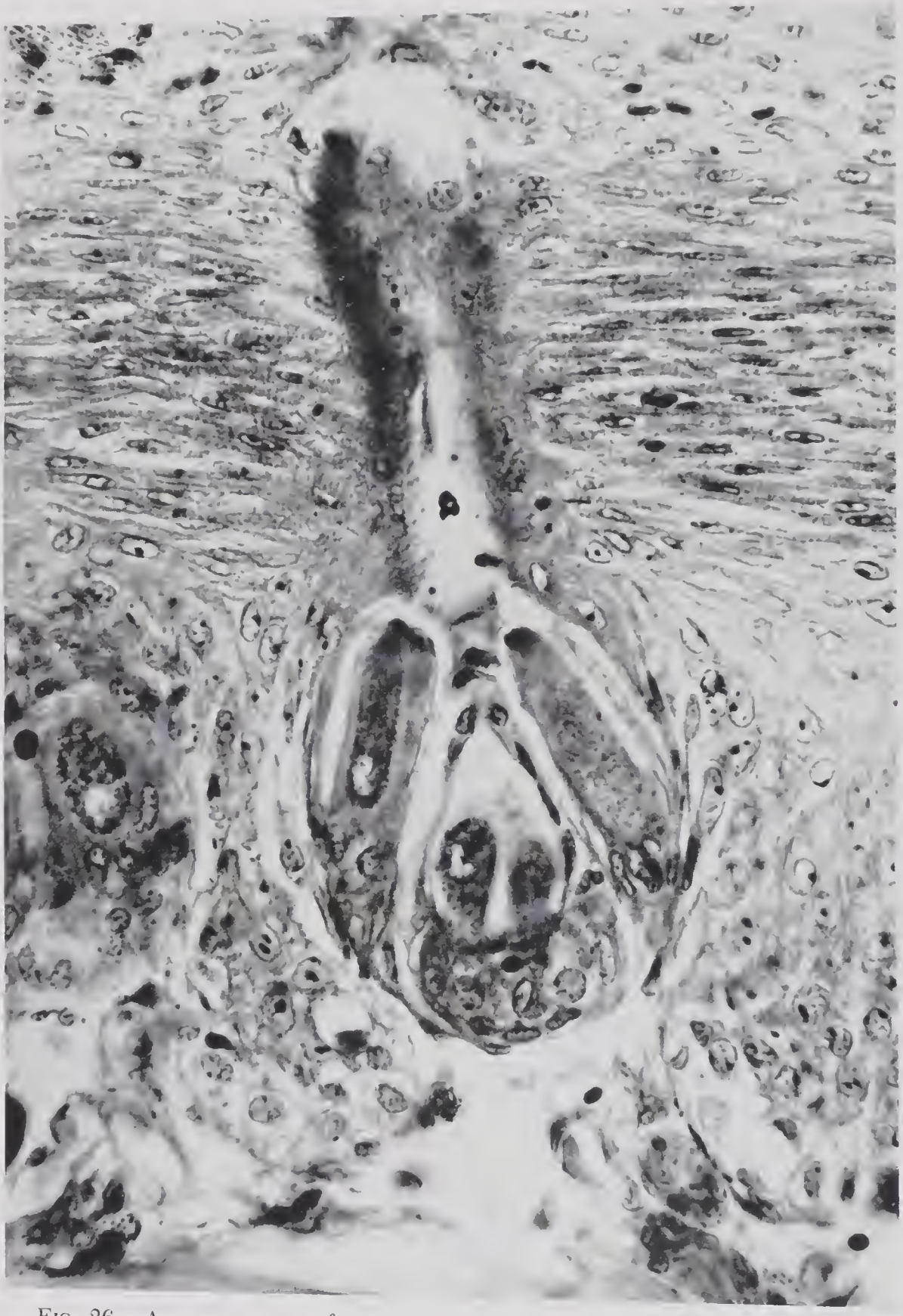

FIG. 26. A mormyromast of type I in the head of *Gnathonemus monteiri*. Several stages of formation and disintegration of pear-shaped sensory cells.

of the mormyromasts can be reasonably grouped in two types, which can be designated as types I and II (Cordier, 1937).

Type I is an intraepidermal ovoid organ, supported on a narrow dermal papilla which enlarges in a hemispheric cup (Fig. 26). The papilla is well vascularized and contains thick nerve fibers and multiple melanophores. From the upper part of the organ a narrow duct leads to the surface of the skin. In the organ itself, two cell types can be dis-

FIG. 27. A mormyromast of type I in *Gnathonemus monteiri*. Silver-impregnated nerve endings envelop sensory cells.

tinguished: (1) narrow cells, in a single layer, tapering in direction of the central lumen; (2) larger, oval or piriform cells which occupy a higher situation in the epithelial lining.

They represent sensory cells, as is clearly demonstrated on silver-impregnated sections; indeed the nerve fiber after reaching the bud divides into two or three branches, which, after losing their myelin sheath, penetrate into the epithelium and expand in a beautiful terminal basket which surrounds the sensory cells (Fig. 27). The most remarkable feature now is the fact that the appearance of the sensory cells is rapidly becoming modified: the cells expand and swell, the nucleus shrinks, and

the cytoplasm shows clear indication of degeneration. Finally the entire cell liquefies, and the remains of this holocrine process are evacuated into the lumen, while new sensory cells are formed. The histological structure therefore becomes more complicated and leads to the aspect of the type II (which corresponds to the descriptions of Stendell and of Franz).

FIG. 28. A mormyromast of type II in *Gnathonemus monteiri*. Numerous neuromastic groups, with sensory cells in different stages of disintegration.

Type II is a tubular glandlike formation of the skin, opening at the surface by a canal generally obturated by an amorphous mucinous plug. The organ is supported on a large dermal papilla in which thick nerve fibers are very conspicuous (Fig. 28).

On the bottom of the organ a continuous process of cell differentiation and cell disintegration takes place: several cell groups can be

distinguished, containing small piriform sensory cells, sustained by elongated supporting cells, while in the vicinity all stages of swelling and liquefaction of sensory cells can be easily followed.

During the whole process the nervous terminal arborization remains intact and, as the organ grows from type I in type II, the course of the nerve fibers changes, becoming incurved from top to bottom, but the aspect of the terminal cup persists. It seems probable, that, after total disappearance of a swollen sensory cell, the terminal nervous arborization will contact newly formed sensory elements. The mormyromasts present thus a unique and interesting cytological feature—the successive differentiation, at the bottom, of neuromastie groups, in which a progressive holocrine transformation of the sensory eells can be followed. The combination of a sensory function with a glandular one is not exceptional; neuromastic sensory cells (see above) in *Protopterus* and *Polypterus* contains secretory globules; and it has been claimed that in the ampullae of Lorenzini, the gelatinous content of the long canals is secreted by the sensory ovoid cells. However, no other example is known of a total and completely holoerine transformation of sensory eells.

(b) The bulbous organs ("*Knollenorgane*"). In addition to the mormyromasts, the skin of Mormyridae contains still some other curious organs which have been described by Stendell (1916) and Gérard (1940).

The bulbous organs are larger then the mormyromasts and are formed by thick epithelial masses that protrude into the dermis. In the center of the organ are two or three large globular cells, while the periphery of the organ is formed by a complex epithelial structure, where it is easy to distinguish supporting cells and young piriform sensory cells. The large central cells are swollen sensory elements, which however do not degenerate as in mormyromasts (Fig. 29). The bulbous organs are endowed with a slow-growing proeessus with lobulation of their contours. Silver impregnation reveals their innervation by thick fibers forming terminal arborizations around the central large cells.

Finally, an interesting observation is reported by Gérard, in the skin of *Mormyrus caballus;* this author found on both sides of the head a few true neuromasts having a typical strueture; in one ease, however, a very conspicuous swelling of the sensory pear cells could be observed, with shrinkage of the nucleus; this picture is very suggestive of a proeess of holocrine liquefaction, very similar, though less pronouneed, to that observed in the mormyromasts.

(c) The maculae laterales. In addition of the peculiar sensory organs located in the skin, the Mormyridae possess, as do other teleosts,

true lateral canals. In these canals lie numerous neuromasts of classical structure, and also true maculae that are broad differentiations of their epithelial lining and differ from normal neuromasts by the fact that they extend longitudinally into the canal for a relatively long distance and do not therefore present the budlike appearance of neuro-

FIG. 29. A bulbous organ ("*Knollenorgan*") of *Mormyrus caballus*. Large swollen cells in the central part.

masts. Moreover, their cytological structure is different. The epithelial thickening of the macula is formed by two cell types, as expected; however, the sensory elements are large ovoid cells with apical hairs extending throughout the entire height of the macula; they are separated by narrow supporting cells with tonofibrils. The pear-shaped appearance of the sensory cell, so characteristic in other endings, is absent. The

macula is covered with a thick and large cupula. Figure 30 is a microphotograph of a macula lateralis of *Gnathonemus monteiri;* the histological structure is very similar to that of the ampullae of Lorenzini of *Plotosus.*

The Mormyridae are thus endowed with a large number of quite peculiar nerve endings. Their innervation by very thick nerve fibers, the presence furthermore of highly developed brain centers belonging to the special somatic component (valvuli cerebelli, area acousticolateralis),

FIG. 30. Intracanalicular macula lateralis of *Mormyrus caballus:* ovoid sensory cells alternate with narrow supporting cells. Compare with Fig. 25.

and the general cytological features of those organs lead one to include them in the sensory system of the lateral line. Concerning their possible function, it should be mentioned that recent physiological investigations (Fessard and Szabo, 1961) have shown that the Mormyridae are endowed with a high sensitivity to electrical stimuli or to electric potential in the environment. As, on the other hand, the Mormyridae exhibit electric organs in the musculature of the trunk, it has been suggested that the skin organs, either mormyromasts or bulbous organs, are the receptors for electrical stimulation. That sensitivity is sufficient to bring to the animal some information on conditions of the environment, or

perhaps on the presence of other fishes of the same species or of different species. The behavioral and positional reactions which the fishes show when electrical stimuli are applied to the external milieu provide very reliable evidence for the specific function of their differentiated skin organs.

2. The Labyrinthine Sensory Epithelia

The labyrinth of vertebrates is a highly specialized sense organ having a rather complex anatomical structure; on the other hand, the cytological features of its multiple sensory areas are remarkably uniform throughout the entire phylum.

The labyrinth is fundamentally formed by two membranous sacs: the utricle and the saccule, filled with a clear fluid, the endolymph.

The utricular part of the labyrinth, shows little anatomical variations in the different classes of vertebrates; it is formed by the utricle itself on which are implanted three (one or two in cyclostomes) semicircular canals, having at one of their ends an ampullar dilatation. The inner epithelial lining is thickened at several regions, in a sensory epithelium. This occurs in each of the three ampullae, where the wall is invaginated in a crista covered with differentiated epithelium. The utricular wall itself bears an oval sensory macula. In addition to these endings, present in all species, there exists, in fishes, a supplementary sensory region, the papilla neglecta, which is more or less regressive in other vertebrates.

The saccular part shows less constant anatomical features and more plasticity. From its fundamental part, the sacculus, arise several expansions, having different degrees of development according to the species considered and each bearing sensory regions like the sacculus itself. Such are (1) the lagena, very conspicuous in fishes, still very apparent in reptiles and birds, and disappearing in mammals; (2) the recessus papillae basilaris, present in amphibians and which in higher vertebrates, crocodiles, birds, and mammals increases and forms the cochlea, while its sensory regions, the papilla basilaris itself, very conspicuous and well developed in birds, presents its highest degree of differentiation in mammals, where it is known as the organ of Corti; and finally (3) an interesting expansion of the sacculus in amphibia, which is described as recessus papilla amphibiorum, with a well-differentiated sensory spot.

The multiple sensory regions of the labyrinthine apparatus respond to the same kind of stimulus, namely movements or vibrations arising in the inner fluid or endolymph. These movements can be produced by changes of position of the head or rotation of the head; the sensitivity is therefore principally proprioceptive. At other points however, the

endolymphatic stimuli are initiated by acoustic vibrations arising in the environment and conducted to the endolymph by means of a more or less complicated system of transmission, including the middle ear, with its bony formations (columella of birds, ear ossicles of mammals); this auditory receptive function is, of course, essentially exteroceptive.

Functionally the sensory apparatus must therefore be separated into a vestibular and a cochlear part. (1) The vestibular sensory part, very uniform in structure and function in all classes of vertebrates, is represented by the maculae of utricle, sacculus, and lagena, by the cristae ampullares, and by the papilla neglecta where present. This part of the labyrinth, proprioceptive in nature, is anatomically characterized by the fact that it is surrounded by a perilymphatic space, filled with perilymph, and having a trabecular structure formed of a meshwork of fine connective trabeculae. It seems plausible to consider this tissue as a kind of damper, isolating the membranous parts and preventing any outer factors from interfering with the movements of the endolymphatic fluids, which should exclusively be elicited by movements of the head. (2) The cochlear part is the auditory apparatus proper. In lower vertebrates this apparatus is poorly developed or possibly entirely lacking, although experimental research has demonstrated the possibility of auditory responses in fishes. The auditory apparatus is anatomically well developed in higher reptiles, birds, and especially in mammals. In order to appreciate a possible acoustic sensitivity of any sensorial regions of the labyrinth, an interesting anatomical feature should be looked for, namely the fact, that the perilymphatic space enclosing the endolymphatic sensorial region must be entirely free, with total disappearance of the meshlike aspect present in the vestibular regions. Furthermore, the limit between endolymph and perilymphatic space which, in the vestibular parts, is a thick basal membrane, is much thinner in the acoustic regions (membrana basilaris of the organ of Corti). Both dispositions are clearly adaptations for an easy transmission of exterior acoustic vibrations to the endolymphatic acoustic endings, by means of this entirely free perilymphatic space.

Such structural patterns are easily seen in the papilla amphibiorum (Fig. 32) and the papilla basilaris of amphibians, which therefore can be considered as the primordia of an organ of Corti.

In several fishes, acoustic transmission is realized by distinct anatomical dispositions, namely the presence of a rostral part of the air bladder in contact with a chain of ossicles which abut on a free perilymphatic space, the latter being separated by a very thin partition from the endolymphatic saecular region in immediate proximity of the macula

sacculi. The necessary anatomical conditions for an acoustic sensitivity are thus fully completed. The anatomical general disposition of the labyrinthine apparatus having thus briefly been recalled (for full details see de Burlet, 1934; Dalcq and Cordier, 1954), the cytological aspects can now be described.

As said previously, the polymorphic and complicated anatomical aspects of the labyrinth contrast with the uniformity of the cytological features. The sensory regions are built on the same general pattern, being formed by two distinct cells types: (1) sensory cells and (2) supporting cells. The sensory cells are generally pear shaped or cylindrical elements with a well-differentiated apical pole, bearing cuticular hairlike formations. They occupy the upper half or third of the entire height of the epithelium, and their basal pole never reaches the basal membrane.

The supporting cells have basally located, dark-staining nuclei, while their cytoplasm is rich in tonofibrils, which can agglomerate in very conspicuous cytological structures such as the pillars of the tunnel of Corti.

Finally each sensory region of the labyrinth is covered by a jelly-like noncellular membrane, in close contact with the hairs of the sensory cells. This membrane is known as "cupula" on the crista ampullaris, as "otolithic membrane" on the maculae, while, in the acoustic regions it is called "membrana tectoria" and is attached to the lateral side of the sensory region.

The general cytological features of the labyrinthine sensory regions this closely resemble those met with in the typical sensory epithelium of the lateral-line system, which were described in Section III,B,1. In recent years, numerous electron microscope investigations have brought further information concerning their fine structure.

a. Vestibular endings. The crista ampullaris is formed by a linear invagination of the wall of the ampulla, oriented perpendicularly to the plane of the semicircular canal itself. The sensory epithelium either covers the terminal ridge of the crista (fishes) or extends also on the lateral declivities. The sensory cells are of the typical pear-shaped or cylindrical type, maintained in the upper part of the epithelium by the supporting cells (Fig. 31). Recent investigations (Wersäll, 1956; Engström and Wersäll, 1958) conducted on the mammalian labyrinth with help of the electron microscope, established the presence of two distinct types of sensory cells: (1) Piriform cells presenting at their base a cup-shaped synapse, formed by a single nerve fiber, which envelopes a large part of the surface of the cell. This nerve ending

contains mierovesicles in small number and is therefore designated as "less granulated" nerve endings. On this cuplike ending abut several other nerve endings, resembling synaptic "boutons" and richly provided with vesieles; these are the "much granulated" nerve endings. (2) Cylindrical eells in contact at their basal regions with numerous synaptic "boutons" issuing from multiple distinct nerve fibers. The double innervation of the erista sensory cells is highly interesting from a physiological viewpoint; it has been suggested that the observed submicroscopical

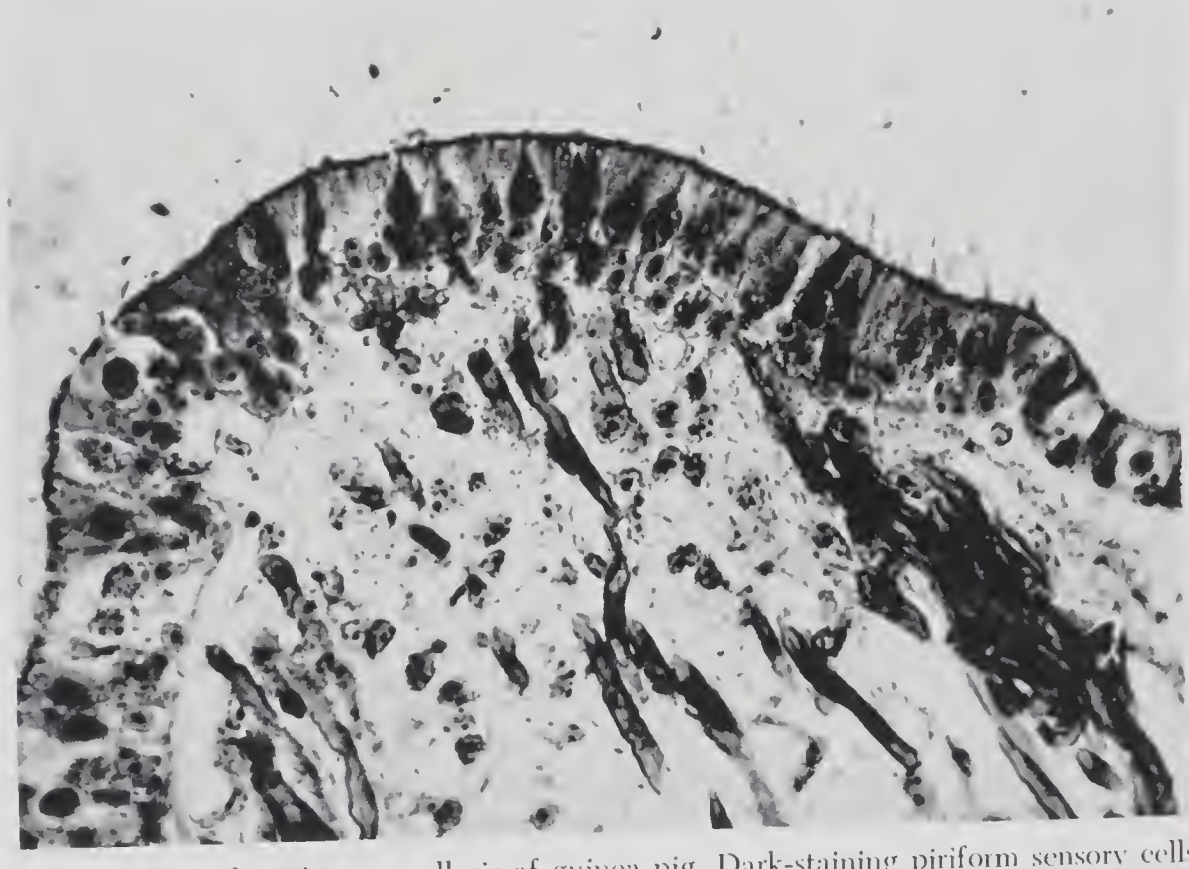

FIG. 31. The crista ampullaris of guinea pig. Dark-staining piriform sensory cells with long sensory hairs. Supporting cells with basal nuclei. Numerous thick nerve fibers in the subepithelial region.

aspects furnish a morphological basis for the existence of efferent and afferent nerve fibers at the level of this sensory ending (Engström, 1958).

Both type of cells contain, in addition to the usual cell organites, numerous microvesicles, located mainly in the apical region, while the apical poles exhibit a cuticular differentiation from which arise numerous long microvilli forming the sensory "hair." These hairs (0.2–0.5 μ) and their tapering extremities can be followed into the jellylike cupula (Fig. 31). Electron microscope observations reveal that in addition to these numerous long and regular microvilli or stereocilia, there exists

in each sensory cell a single true cilium (kinocilium) showing on transverse section the typical ring of nine groups of protofibrils.

The macular regions of the labyrinth present a cytological structure which, in its essential features, is similar to that of the cristae although its finer detail have been less attentively studied thus far (Wersäll *et al.*, 1954).

The main histological difference between cristae and maculae is the presence in the former of a cupula, a jellylike structure covering the

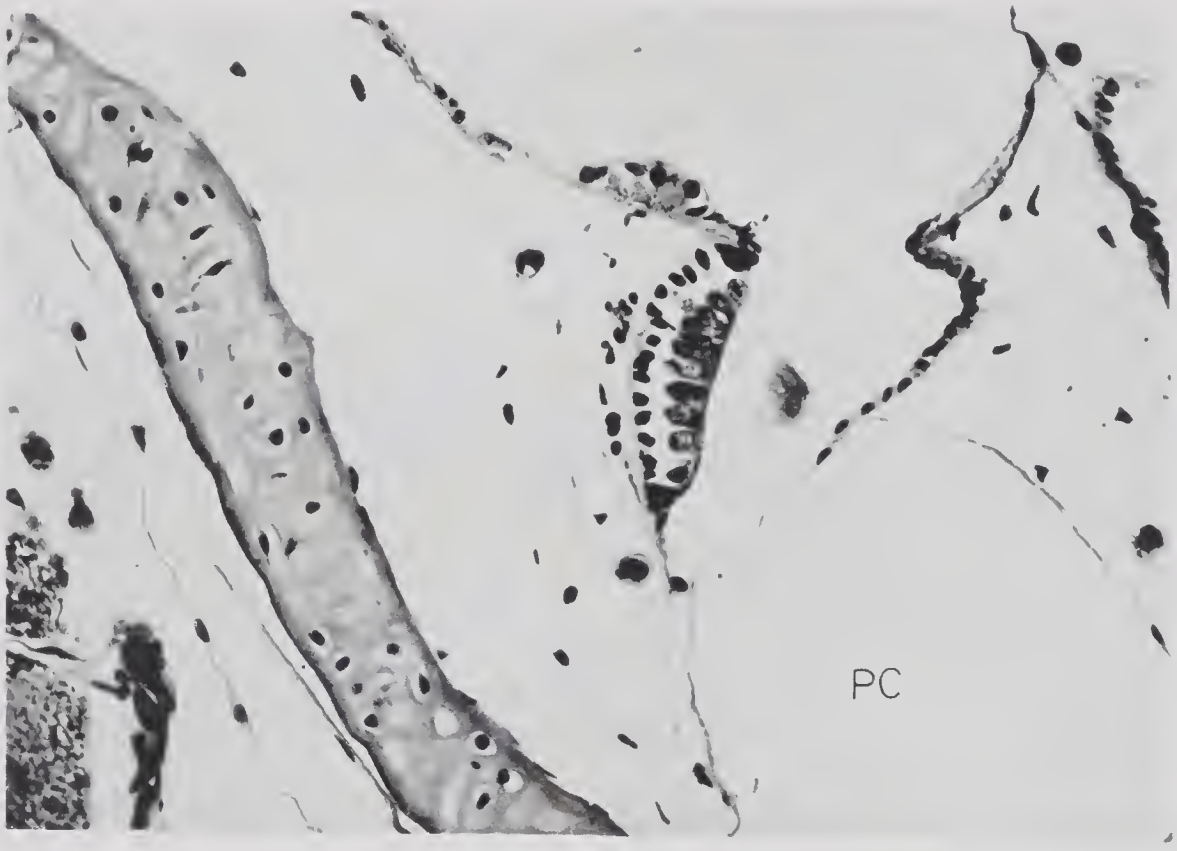

FIG. 32. The papilla amphibiorum in *Proteus*. The receessus is separated by a thin membrane from the perilymphatic canal (*PC*), which is entirely free from connective meshes. The papilla shows piriform sensory cells and a tectorial membrane.

sensory region, while in the macula a similar formation exists, encrusted with inorganic salts, and known as otolithic membrane. In fishes, true otoliths exist on the maculae (sacculolith or sagitta, utriculolith or lapillus, lagenolith or asteriscus) presenting characteristic anatomical features, varying according to the species and to the age of the animal and of great interest from the zoological and paleontological standpoint. It may be recalled here that otolith-like formations have been discovered in some terminations of the lateral-line system as in the maculae laterales of *Polypterus* (Fig. 19) and in the organs of Fahrenholz of *Protopterus*.

b. Acoustic sensory epithelia. The acoustic regions of the labyrinth, being the papilla amphibiorum, the papilla basilaris, and the organ of Corti, present the same general cytological features and are formed of cylindrical or flasklike sensory cells, and more or less differentiated supporting cells with tonofibrils. Figure 32 shows in its simplest form a terminal of this kind, the papilla amphibiorum of *Proteus*. The recessus of the papilla terminates with a thin wall, in contact with a perilymphatic duct entirely free from connective meshes. The sensory region shows a few typical piriform sensory cells and is covered with an amorphous

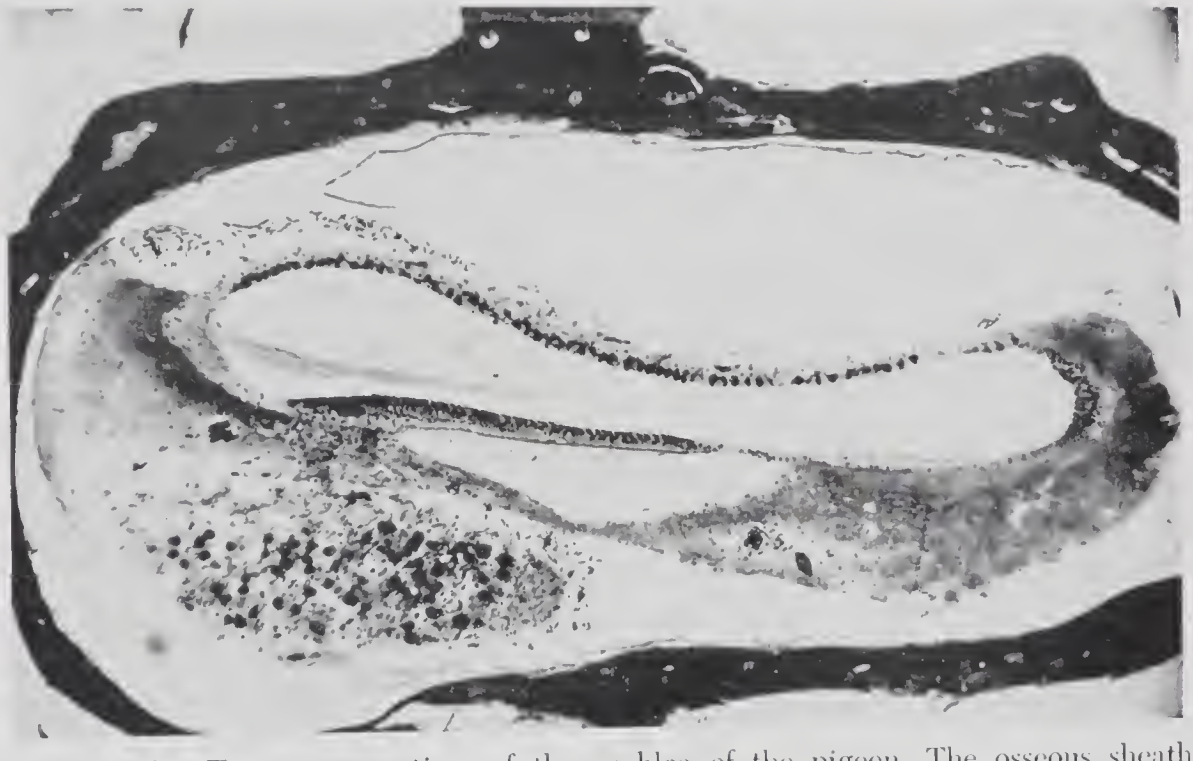

FIG. 33. Transverse section, of the cochlea of the pigeon. The osseous sheath encloses the membranous cochlea, with its supporting chondroid tissue and the ganglion of Corti. The elongated organ of Corti occupies the inner part of cochlea. Inner hyaline cells and homogeneous cells are seen, a long tectorial membrane being firmly attached to the latter.

membrane, which is really attached to the dorsal wall of the recessus and is therefore a true membrana tectoria.

In higher reptiles and in birds, the papilla basilaris, present in amphibia, enlarges and forms an incurved tubular expansion, the cochlea, located in a similarly formed osseous sheath from which it is separated by a perilymphatic space. The cochlea bears on its convex side a long sensory area, which is represented in Fig. 33. The area is composed of numerous sensory cylindrical cells, with terminal sensory hairs, separated and maintained by narrow, supporting cells, containing bundles of tonofibrils which terminate at the apical pole in a clublike swell-

ing, while their basal regions form a loose trabecular structure through which the afferent nerve fibers run, in the direction of the sensory cells (Fig. 34).

The papilla basilaris is entirely covered by a membrana tectoria, which is attached on the lateral side of the papilla; at this level there are no sensory cells, the epithelium being formed by the so-called "hyaline cells," near the sensory region and the "homogeneous" cells a little beyond; the latter are the origin of the membrana tectoria, which is firmly attached to their apical pole.

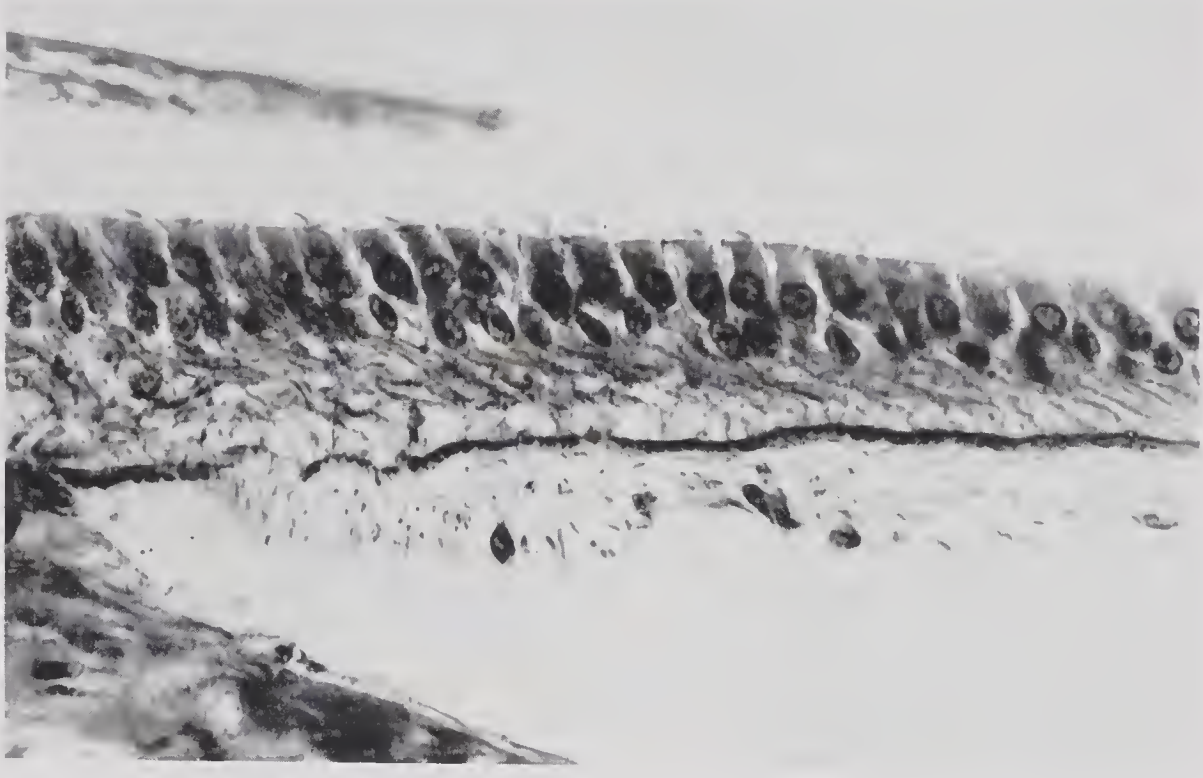

FIG. 34. The organ of Corti of the pigeon. Cylindrical sensory cells with incurved auditory "hairs" occupy the upper third of the organ. Note supporting cells with tonofibrils ending in small apical bulbs, and loose meshlike basal cytoplasm.

In mammals, the papilla basilaris is represented by the highly differentiated organ of Corti, and its recessus forms the ductus cochlearis. Descriptions of this sensory organ are given in full detail in any textbook of histology and are therefore of common knowledge. It will be sufficient to remember, with respect to cytology, that two cell types are easily identified; the supporting cells present different degrees of differentiation, the most remarkable being the formation of pillar cells, outer and inner, limiting the so-called tunnel of Corti, while the sensory or auditory cells or hair cells are in a single row at the mesial side of the tunnel, and in 3 or 4 rows at its lateral side. Other cytological aspects of

supporting cells are the Deiters cells, which have enlarged apical poles, the phalanges forming a limiting membrane enclosing closely the apical poles of the auditory cells. Hensen and Claudius cells are nonsensory elements located still farther laterally.

The cytology and fine structure of the auditory cells have been investigated by several authors (Smith and Dempsey, 1957; Spoendlin, 1958, 1959; Engström and Wersäll, 1958; Iurato, 1961; Smith and Sjöstrand, 1961) by means of the electron microscope. These observations bear generally on the cochlea of rodents.

There is a definite difference in outer form between the inner and the outer auditory hair cells (Fig. 35). The inner hair cell is polyhedral and irregular in outline whereas the outer cells are more regularly prismatic, having a hexagonal apical pole wedged between the hexagonal apical phalanges of the Deiters cells.

Different superposed regions can be identified in the auditory cells, as stated by Iurato (1961): (1) The apical zone has a cuticular thickening in which the auditory hairs are implanted. Each hair, 4–5 μ in length, consists of an electron-opaque fibrillar axial core, surrounded by a less dense material. The extremities of the hairs abut on the tectorial membrane, but do not penetrate into it. No true cilium can be observed. (2) The intermediate zone contains numerous microvesicles, mainly arranged near the surface; mitochondria and Golgi material are also present. (3) The perinuclear zone is poorer in cell organelles. (4) The basal synaptic region shows on the inner hair cells, as on crista cells, a double innervation represented by "less granulated" and "much granulated" nerve terminals, whereas the endings observed at the base of the outer cells are chiefly of the "much granulated" type. It has been suggested that the much granulated endings could represent the terminals of efferent fibers, the vesicular material being identical with synaptic vesicles as observed in presynaptic position on efferent endings. The less vesiculated endings would therefore be the true afferent endings. Anatomical and physiological research has indeed demonstrated the existence of efferent nerve fibers, originating in the superior olivary complex and reaching the cochlea, where they form the helicoidal bundle of Rasmussen (1942, 1953) in the lamina spiralis. However, this bundle is small in comparison to the bulk of afferent dendrites of the ganglion of Corti, which represent the sensory fibers; the predominance of highly granulated nerve terminals on the hair cells seems therefore highly contradictory to their interpretation as endings of the Rasmussen bundle.[1]

[1] An afferent cochlear bundle has been described by Boord (1961) in caiman and pigeon. Its precise mode of termination on the organ of Corti is thus far unknown.

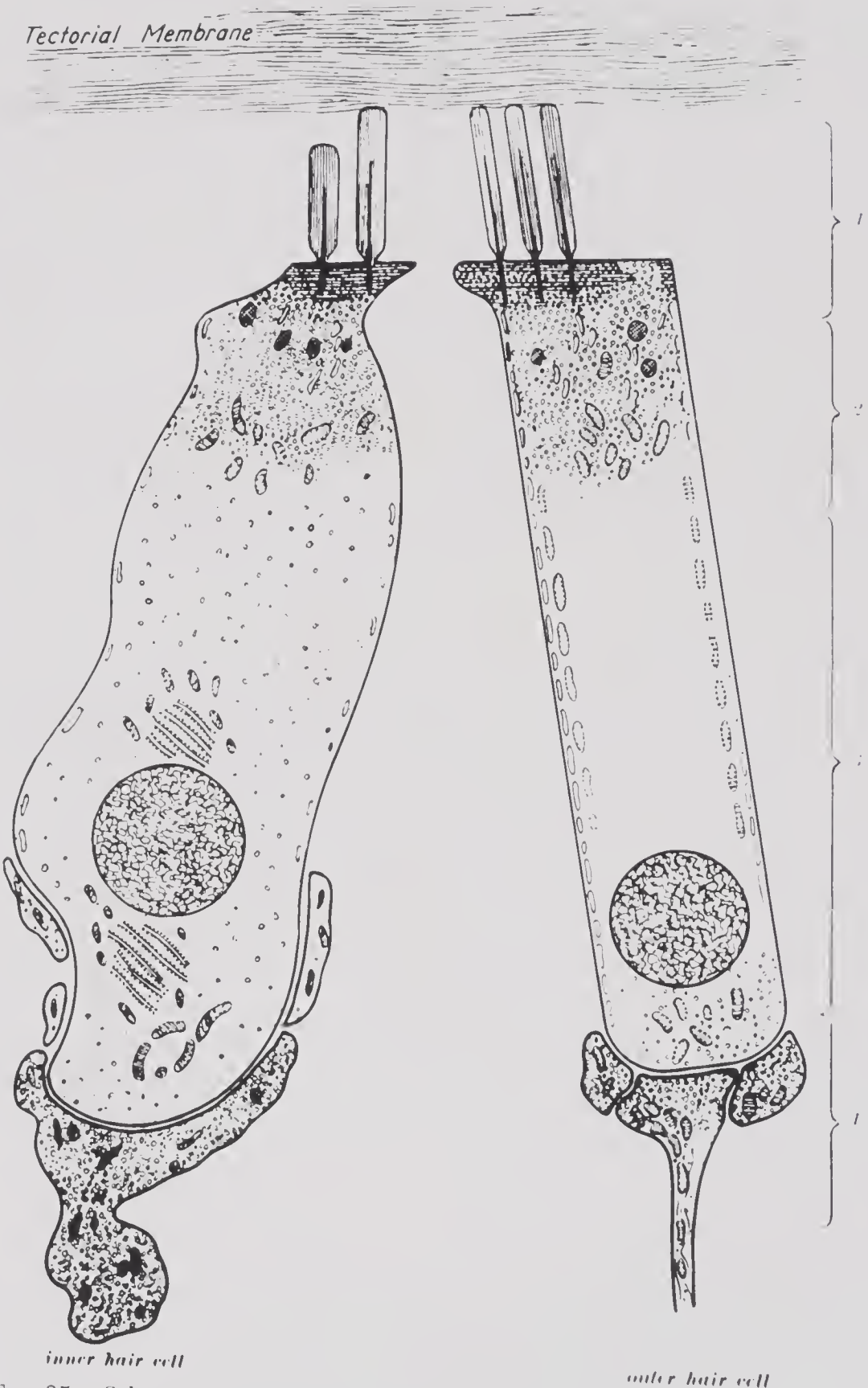

FIG. 35. Schematic drawing of inner and outer auditory hair cells in organ of Corti of the guinea pig. *1*, Dense apical region with "hair" abutting on the tectorial membrane; 2, upper region with microvesicles, often located near the cell membrane; 3, perinuclear region; *4*, basal region with "much" granulated and "less" granulated nerve endings. Electron microscope. From Iurato (1961).

The Rasmussen bundle, as shown by electrophysiological research (Rasmussen, 1960; Desmedt, 1960) represents a centrifugal system for control of acoustic sensitivity, and its stimulation depresses acoustic responses. It has been suggested that this effect is produced by a chemical inhibitory transmitter released from the axon terminals. Thus far no direct chemical proof of this interpretation has been brought. However, it should be mentioned that a positive cholinesterase reaction has been obtained (Schuknecht *et al.*, 1959) at the base of the auditory cells of the cat, although this result has probably no meaning for the explanation of the referred physiological observations.

At the base of the outer and inner hair cells of the organ of Corti of the guinea pig, Smith and Sjöstrand (1961), using the electron microscope, found a characteristic structure at the level of the synaptic junction. A presynaptic dense ribbon surrounded by numerous vesicles is present in front of the synapse. This structure seems identical with the one described later by Barets and Szabo (1962) in the ampulla of Lorenzini of Torpedo (see Section III,B,1, Fig. 25A).

Extensive histochemical investigations on the cochlea of the guinea pig have been made by Vinnikov and Titova (1961–1963) with a technique for isolation *in vitro* of the organ of Corti. Cytochemical methods for demonstration of different biological substances have been applied either to the organ at rest or after direct acoustic stimulation. In the auditory cells acetylcholinesterase, acid and alkaline phosphatase were found in the apical hairs, while cytochrome oxydase and succinic dehydrogenase were present in the mitochondria. Glycogen and protein-bound SH- and COOH-groups have been identified. Quantitative variations of the different enzymes and substances, in relation with the frequency of the acoustic stimulus and its duration, have been observed. On the basis of their findings the authors propose a cytochemical theory of hearing, according to which the couple acetylcholine-acetylcholinesterase present in the hairs is of great importance for the response of the sensory cell to sound.

IV. Pseudosensory Cells (Sensory Cells *Largo Sensu*)

As stated in the introductory considerations of this chapter, in a great number of sensory receptors, however discriminating and epicritical their receptivity may be, true sensory cells are lacking. The stimulus is received by the terminal dendrites of the ganglion cells; the nerve fibers can terminate freely among the innervated tissues, or be in close contact

with some cellular elements which constitute then an intrinsic part of the anatomy of the organ. Such cells can be epithelial, muscular, connective, or lemnoblastic (Schwannian). It seems dubious that they are directly involved in the transducing of the stimulating energy into nervous impulse; nevertheless they must play a necessary role in the function of the organ involved. We suggest therefore to designate them as pseudosensory cells or sensory cells *largo sensu*.

The large group of sense organs built on this type includes the multiple and diversified structures responsible for cutaneous sensitivity (tactile sensations, pain, heat, and cold) and for sensitivity of viscera to pressure or distention, the stretch receptors of musculature, the chemoreceptors for blood homeostasis, etc.

The histology of these organs is well known, and some of them have been the object of histochemical observations or electron microscope observations. In absence, however, of true sensory cells, their description lies outside the limits of this chapter.

Nevertheless, in order to present a more complete survey of the morphological diversities of sensory organs, it has appeared reasonable to present two examples of sensory organs chosen for their interesting histological and cytological features and also for the fact that they do not commonly appear in classical descriptions of receptory organs. These two structures are, first, the organ of Eimer in the snout of the mole, and, second, the pit organ of pit vipers, which is a remarkably organized thermoreceptor. In both these organs, cellular elements which can be considered as pseudosensory cells have been described.

A. The Organ of Eimer of the Mole

Among the cutaneous sensory endings of mammals, the organ of Eimer of the mole (*Talpa europaea*) occupies an interesting and rather exceptional position, since it combines in its complex structure the different aspects which are observed in the nerve terminals of the skin. Indeed, in the organ of Eimer are present: (1) free intraepidermal nerve endings; (2) so-called "tactile" cells of Merkel; (3) lamellar intradermal corpuscles.

The organ of Eimer represents furthermore a sensory ending exhibiting the special sensitivity which has been designated by Edinger as the "oral sense," being the concentration of tactile nerve endings in the vicinity of the mouth. In this group we find the tactile hairs or vibrissae present in many mammals, the lamellar corpuscles found in great abundance in the bill of waterfowl, the nerve terminals present in the tongue

of some higher vertebrates (as in the anteater, *Myrmecophaga*, or in the woodpecker), etc.

The snout of the mole is a fleshy protruding part of the head having a coarse granulated surface. A section through this region provides a histological picture not very dissimilar from that observed in the human

FIG. 36. Organ of Eimer of *Talpa europaea*. Central cellular column surrounded by nerve fibers (appearing as clear canals). Two Merkel cells lie at the base of the organ, with nervous disk at the base (clear space).

fingertips. Broad epidermal papillae, with flattened basis and corresponding to the superficial bulging profiles, alternate with small tapering papillae. The former are similar to the intermediate ridges of the human digital skin (glandular ridges), while the narrow ones correspond to the cristae limitantes. In the small papillae, the basement membrane of the epidermis is plicated, thickened, and firmly anchored in the dermis, whereas in the broad papillae the basal membrane is smooth and thinner.

In each of the broad papillae, there exists an organ of Eimer appearing as a longitudinal column formed, in the axis of the papilla, by the superposition of regular cuboidal cells in 2 or 3 rows and reaching from the base of the papilla to its surface. At the base of the column exist 2 or 3 clear cells, with long axis parallel to the skin surface and having a long transverse nucleus with irregular contour. These cells are identical with the "tactile" cells of Merkel, described in numerous sites of the mammalian epidermis (Fig. 36).

Abundant nerve fibers penetrate the Eimerian papilla, after losing their myelin sheath at the dermoepidermic limit. Some of them form a kind of circular grate around the central column while others, somewhat thicker, run in the axis. These ascending fibers can easily be followed on nonimpregnated sections (Fig. 36), where they appear as clear lines. The fibers run in the intercellular spaces, as can easily be seen on transverse sections. Boeke (1933) in his classical description of the organ of Eimer, represents the fibers as intracellular, in a figure often reproduced in textbooks. This interpretation is of course erroneous.

Other nerve fibers, penetrating at the base of the organ reach the basal tactile cells and form a discoidal expansion at their proximal surface.

The epidermal part of the organ of Eimer is now completed by a dermal component. Immediately beneath the base of the papilla, and in close vicinity to it, 1–3 lamellar corpuscles can be seen, resembling small Pacinian corpuscles.

A short thick nonramified nerve fiber is visible in the central core oriented parallel to the skin surface, while the bulk of the corpuscle is formed by superposed lamellae. At the periphery a few nuclei are seen, whereas the central part of the corpuscle is devoid of nuclei (Fig. 37).

In the complex organization of the organ of Eimer, two structures could possibly be included in the category of pseudosensory cells as defined before, namely, the Merkel cells and the lamellar cells of the corpuscle.

1. Merkel Cells

The Merkel cells have been known in classieal histology for a very long time as ovoid or almond-shaped eells present in varying loeations in the epidermis of mammals and also in the outer epithelial sheath of the hair. Examined with the light microseope, their eytoplasm appears as definitely different from that of normal epidermal eells, being elear and deprived of tonofibrils. The characteristie feature, however, is the

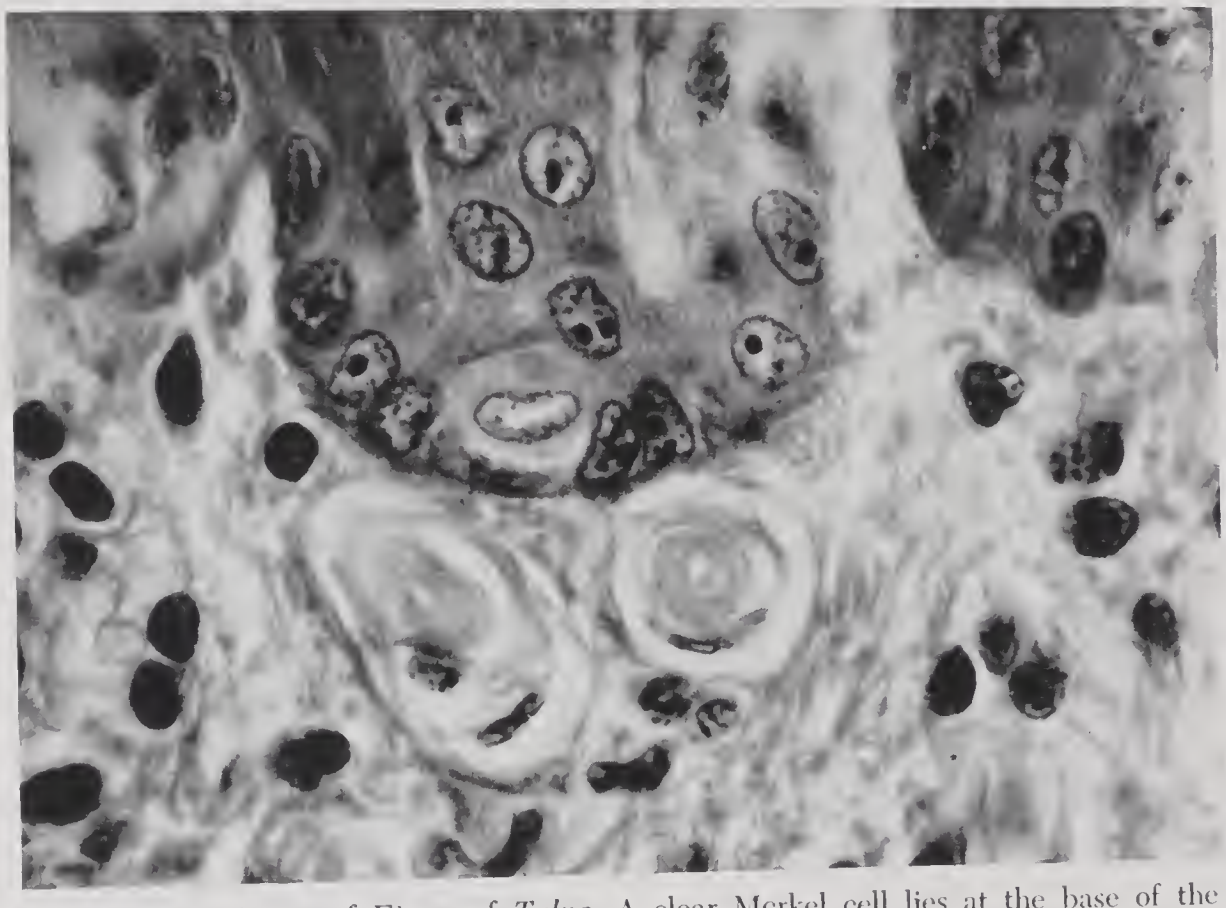

FIG. 37. Organ of Eimer of *Talpa*. A clear Merkel cell lies at the base of the epidermal column; two lamellar corpuscles in the dermis.

presence of the nervous disk in close eontaet with the inferior surface; the disk is easily seen without silver impregnation (Figs. 36 and 37) and, contrary to Boeke's opinion, there is no penetration of nerve fibers nor neurofibrillar material in the cytoplasm of the Merkel eell. On eleetron microscope photographs (R. Cordier and P. Drochmans, unpublished data), the Merkel cell is easily identified, by its elongated transverse nucleus (Fig. 38). Furthermore, its surface presents a number of dendritic processes which ean be followed into the intercellular spaees which surround the cell on nearly all sides. A conspicuous centriole can be found, and the nervous disk is seen at the base, with its numerous

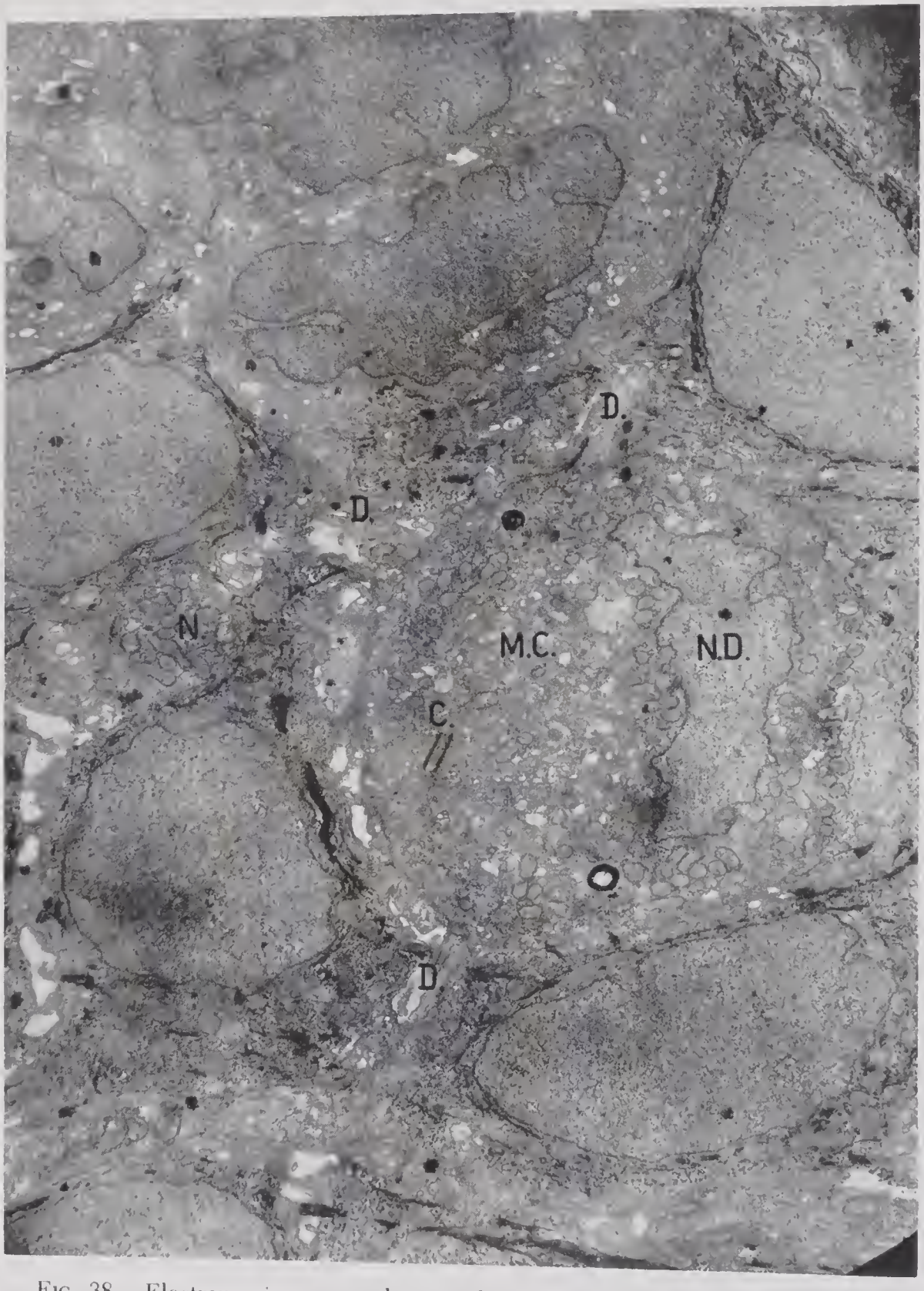

FIG. 38. Electron microscope photograph of the base of the epidermal column in the organ of Eimer of the mole. Orientation of the picture: dermoepidermal limit at the right side, surface of skin at the left. Merkel cell (*M.C.*) with centriole (*C*), nucleus (*ND*), dendritic process (*D*). *N*, intraepidermal nerve fiber. EM Siemens. Magnification: × 7,962. From Cordier and Drochmans (unpublished data).

mitochondria. Tonofibrils are lacking. The general aspect of the Merkel cell suggests a dendritic melanoblast.

Furthermore it is not very dissimilar from the characteristic "tactile" cells present in the corpuscle of Grandry of birds, which, like the Merkel cells, are clear ovoid or flattened cells in close contact with a discoidal nervous terminal. Therefore it seems by no means certain that the Merkel cell is simply a modified epidermal cell. The hypothesis of its Schwannian nature should be considered, although the definite proof of this is difficult to bring. Merkel cells, melanoblasts, and tactile cells of the corpuscle of Grandry, in this case, would represent derivates of the neural crest.

The characteristic innervation of the Merkel cell by the nervous disk warrants the conclusion that it intervenes in the perception or transmission of some exteroceptive stimulus; however, it cannot be considered as a true sensory cell as defined before, and no information exists on its precise role.

2. *Lamellar Corpuscles*

The lamellar corpuscle, or Eimerian corpuscle, presents a close resemblance to other lamellar nerve endings described in the skin of higher vertebrates, such as corpuscles of Herbst, Pacini, Krause, Meissner. An electron microscope investigation (Cordier and Drochmans, unpublished data) has revealed a structure very similar to that observed in Pacinian corpuscles by Pease and Quillan (1957) or in Meissner corpuscle by Cauna and Ross (1960). The corpuscle is formed of cells having peripherally located nucleated bodies, while their cytoplasm spreads out in long and narrow lamellae that surround in multiple superposed layers the inner core. Here the nerve fiber is seen with its characteristic richness in mitochondria. The lamellar cells present some interesting cytoplasmic features. In their nucleated body, very conspicuous filaments are present, closely similar to neurofilaments, while in the lamellar expansions a great abundance of small vesicles of about 300 A. is observed. The vesicles are mainly numerous in the immediate vicinity of the cell membrane.

The cytoplasmic lamellae, which form the bulk of the corpuscle, are separated by narrow interlamellar spaces, where collagen fibrils, with characteristic periodicity, and reticular fibrils are present in great quantity. At multiple points of the cell surface, the vesicles open freely into the interlamellar spaces (Fig. 39). It is highly probable that they represent images of a process of micropinocytosis and are not to be identified as synaptic vesicles. No direct synaptic junction has been ob-

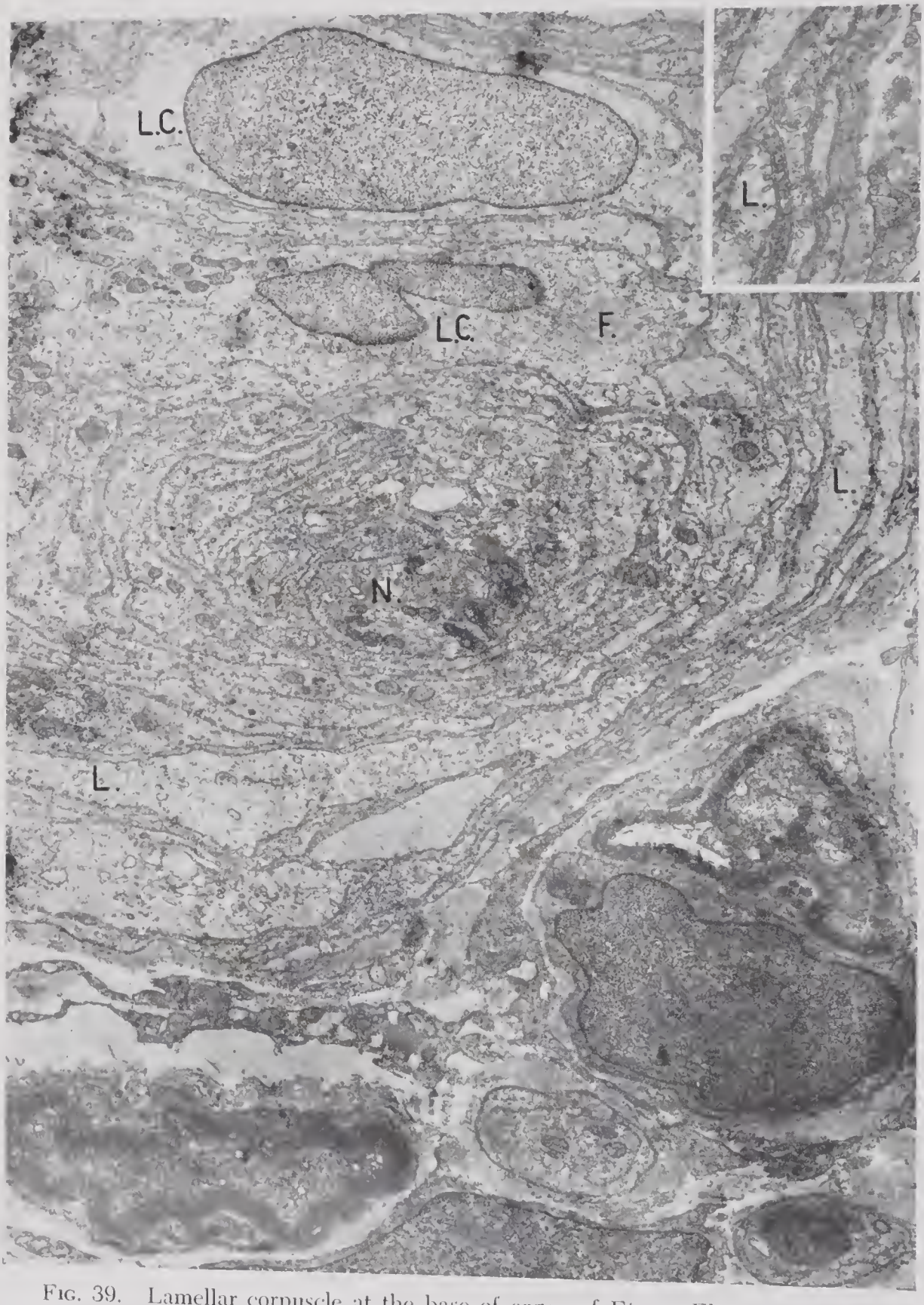

FIG. 39. Lamellar corpuscle at the base of organ of Eimer. Electron microscope photograph. *N*, Axial nerve fiber; *LC*, lamellar cell with nucleus and fibrils (*F*); *L*, lamellae separated by interlamellar spaces. Inset at upper right: enlarged region showing lamellae with micropinocytotic vesicles and interlamellar spaces with collagen and reticulin fibrils. From Cordier and Drochmans (unpublished data).

served between the central nerve fiber and the innermost lamella in the Eimerian corpuscle, although a synaptic thickening of the two cell membranes in contact at this level has been described by Cauna and Ross (1960) in the Meissner corpuscle. As in other lamellar corpuscles, it seems reasonable to consider the lamellar cells as Schwannian elements, enclosing and protecting the inner nervous core; they certainly have a prominent meaning in the function of the corpuscle, but their true sensory nature is dubious. Therefore, we place them in our group of pseudosensory cells.

Finally, it should be mentioned that some information has been brought recently on histochemical aspects of lamellar corpuscles.

A positive cholinesterase reaction has been obtained in the inner bulb of the Pacinian corpuscle (Quilliam, 1958) and of the Herbst corpuscle (Winkelmann and Myers, 1961) and in the interlamellar spaces of the Meissner corpuscle (Cauna, 1960; Winkelmann, 1961). Cauna and Alberti (1960) furthermore, investigated the organ of Eimer, but, very surprisingly, although a cholinesterase reaction was obtained in the intra- and subepidermal nerve fibers, neither Merkel cells nor lamellar corpuscles could be found in the material examined.

B. The Pit Organ of Pit Vipers

It has been known for a long time that snakes belonging to a subgroup of Viperidae, namely the Crotalinae, or pit vipers, exhibit on both sides of the head between nostril and eye, a depression lined with cornified epithelium, at the bottom of which exists a highly innervated membrane. The function of the organ has been recently investigated by physiological methods (Bullock and Diecke, 1956), which demonstrated its sensory nature as a thermoreceptor. The pit organ presents a high sensitivity to infrared waves, between 15,000 and 40,000 A., as tested by accurate electrophysiological observations. The histological structure of the organ has been studied previously by West (1900) and Lynn (1931) and, with greater accuracy, by Bullock and Fox (1957).

A section through the pit organ (Fig. 40) shows that it is really formed by two superposed cavities, the superior being the depression visible at the outside, while the inferior chamber is a lateral invagination of the skin, in free communication with the surface by a bifurcated canal opening on the one side in front of the eye, and on the other side in the anterior and superior part of the pit.

The membrane forming the bottom of the pit is covered on both sides by a thin layer of cornified epidermal cells (Fig. 41). Between them can be seen some connective tissue, numerous blood vessels, and very

FIG. 40. Total view of the pit organ of *Agkistrodon mocassen,* showing the superior and inferior chambers and the sensory membrane.

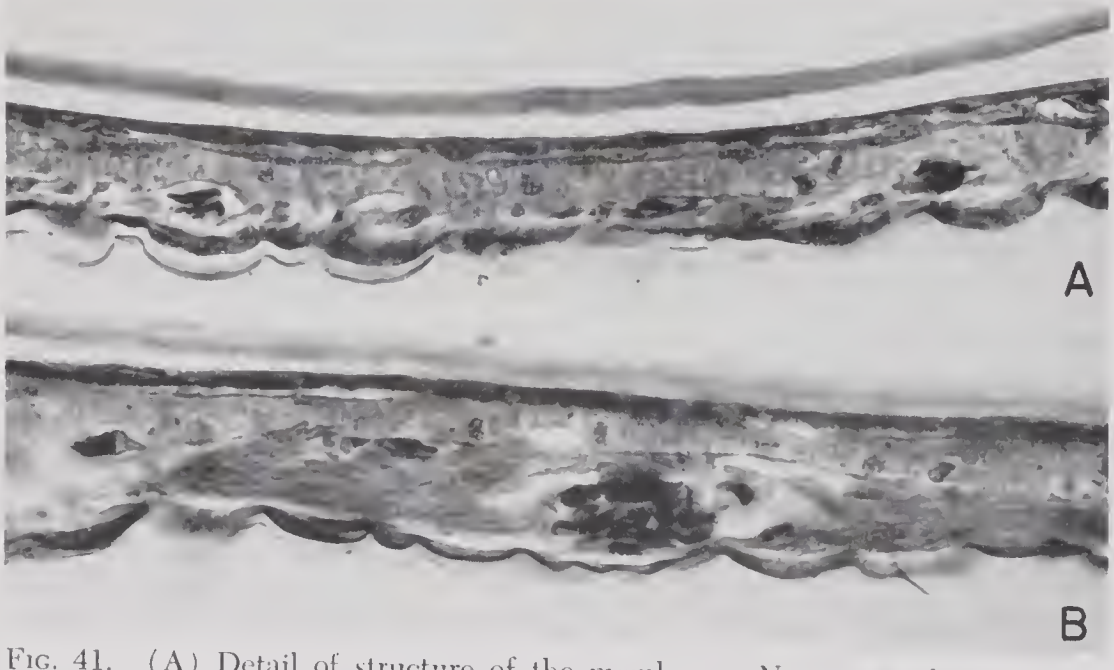

FIG. 41. (A) Detail of structure of the membrane. Narrow epidermal cornified layers on both sides. "Parenchyma" cells with granular cytoplasm and small nuclei. (B) The same membrane showing the localization of the nerve fibers between "parenchyma" cells and inner epidermal lining. A distinct cell membrane can be seen between nerve fibers and parenchyma cells.

abundant nerve fibers (Fig. 41). The bulk of the organ, as observed with the light microscope, is formed by a layer of large cuboidal cells, with small irregular nuclei, and a coarse-granulated aeidophile eytoplasm. At the periphery of the membrane, this layer continues for a short distance into the dermis, and some of these granular cells ean be found scattered throughout the dermis, sometimes far away from the insertion of the membrane in the projeeting lips which limit the external opening of the pit. Bulloek and Fox describe these cells as "parenchyma" cells. From observations with classical methods of light microseopy, it seems therefore evident that the parenehyma cells are modified dermal eells, somewhat resembling histiocytes, whieh form a continuous epithelial layer in the pit membrane. The nerve endings represent a remarkable morphology, as observed in total preparations of silver-impregnated membranes (Fig. 42). The nerves which are located between the inner layer and the parenchyma cells, after losing their myelin sheath, expand in very flat broad palmate structures from whieh several processes branch, terminating in fine free endings. As described by Bullock and Fox, the palmate ending resembles that of a multipolar neuron without nucleus. The elose eontact between nerve terminals and parenchyma cells leads to regarding the latter as sensory cells; however, no proof exists of their receptory nature. Although their exaet origin and significanee remain dubious, the observations made until now permit plaeing them in the general group of pseudosensory cells (sensory cells *largo sensu*) as defined previously.

A reeent investigation of the pit membrane of Crotalidae has been made by Bleichman and de Robertis (1962) with the electron microscope and has led to different conclusions on the nature of the "parenchyma" cells. The very existence of such cells is formally denied on the evidence of electron microseope pictures. Indeed the bulk of the membrane is oceupied by amyelinated nerve endings containing enormous quantities of mitochondria. It is stated that half the volume of the membrane is made of eompact masses of mitochondria. The nuclei of the so-called parenchyma cells belong in reality to small thin vaeuolated cells with a flat processus extending beneath the outer epithelium, but which in no case form a continuous layer.

We find thus a total discrepancy between light and electron microscope observations. If the electron mieroseope observations are to be aceepted, then no sensory elements of any kind exist between the stimulus and the receptory endings; it is suggested that the mitochondria themselves, crowded in large quantities beneath the surfaee, could be involved in the mechanism of transducing temperature changes in neural

activation, perhaps by means of modifications of the enzymatic activity and the ionic conditions of the membrane.

The pit organ constitutes a most interesting organ which, apart from its remarkable intrinsic physiological and histological features, brings us

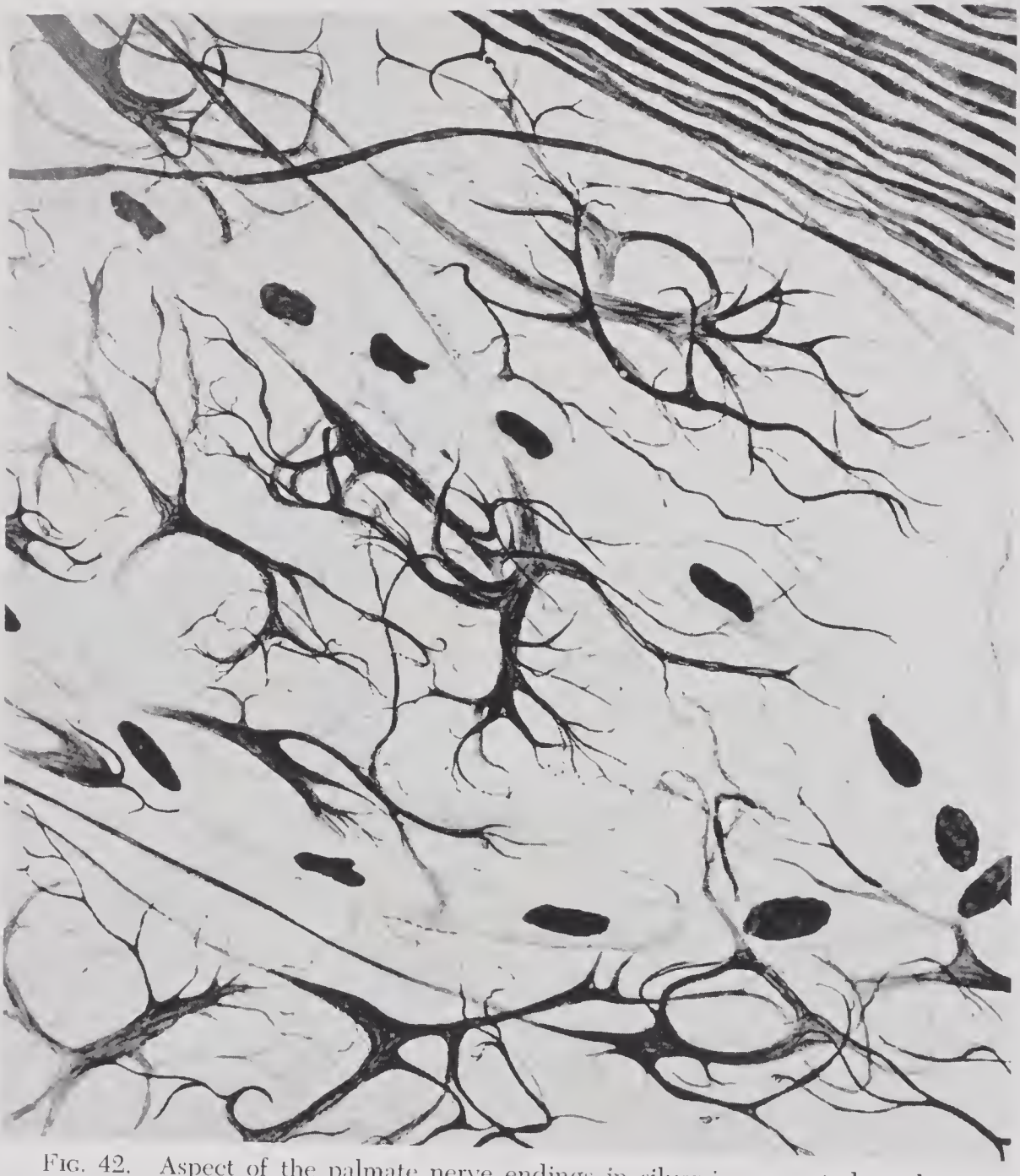

FIG. 42. Aspect of the palmate nerve endings in silver-impregnated total mount of membrane. From Bullock and Fox (1957).

an example of total discrepancy between results obtained either by classical or by new observations methods. Indeed, no histologist, looking with the light microscope at sections from well-fixed and correctly stained material, could have the least doubt as to the existence of the epitheloid

parenchyma cells, with their granular cytoplasm and small dark irregular nuclei. If, on the other hand, the results of electron microscope observations are accurate, the former interpretation is entirely illusory and erroneous. In this case, what must we think of the scattered dermal granular cells which are present in the dermis close to the opening of the pit and which, in their cytologic features, are identical with the parenchyma cells of the membrane?

V. Conclusions

From the general survey of the structure of sense organs and sensory cells, a few concluding remarks are warranted. In former decades, histophysiological research aimed at establishing the relationship between function of organs and tissues and their structure. In the same trend of thought, modern cytology must hope, as its principal achievement, to find, in the observations of the minute and submicroscopical structures of the cell, a suitable basis for the understanding of its main functions.

Very important progress has certainly been made in this direction. In gland cells, the intervention of multiple cell organelles, such as endoplasmic reticulum and ribosomes and Golgi apparatus, in the elaboration of the visible products of secretory activity has been amply demonstrated. In muscle cells, the microscopic aspects of the myofibrils and protofibrils, in resting stage and during contraction, have been closely related to the behavior of the specific proteins that are responsible for muscle contraction.

With respect to sensory cells, the actual results are certainly less satisfactory. The function of sensory cells is to perceive stimuli of varied nature, mechanical, chemical, physical, thermic, or electric, and to transduce the energy received into a nervous impulse. What parts of the cell structure, what cell organelles, are implicated in this process?

It has been claimed (Bairati, 1960) that, for the capture of the specific stimulating energy, sensory cells are endowed with specifical apical structures in direct contact with the stimulus.

And, indeed, in neurosensory cells as in true sensory cells, the apical pole bears either more or less modified cilia (as in olfactory cells and ependymal cells) or cuticular formations and sensory "hairs" (as in lateral-line organs and labyrinthine endings), or more or less developed microvilli (as in gustatory cells). However, such structures can be found in nonsensory cells, as in intestinal, renal, epididymal, and respiratory epithelia. Furthermore, highly differentiated and discriminative nerve receptors, such as the multiple nerve endings for cutaneous sensitivity

or for deep sensitivity, are not endowed with true sensory cells, and in the morphological structure of the elements implicated in the perception of the stimuli, no such apical differentiations exist.

A more promising aspect of sensory cells is the presence, in most of them, of submicroscopical vesicles often located near the cell membrane (olfactory, gustatory, auditory cells). It is very tempting to assimilate these cell organelles to the synaptic vesicles described in the efferent terminals, in presynaptic position. In sense receptors, as for instance in the organ of Corti, microvesicles are found both in the sensory cells and in the efferent nerve terminal, i.e., in pre- and postsynaptic position. The intervention and role of the microvesicles at the level of sensory synapses is of course unknown. In efferent synapses, especially in the neuromuscular junction, it has been suggested that the presynaptic vesicles could represent the material support of the known chemical transmitter. But no such transmitter has thus far been identified with certainty in the afferent synapse.

Histochemical research, and especially histoenzymatic research is no less disappointing. The detection of different enzymes at the level of the gustatory terminals leads to highly doubtful hypotheses. Cholinesterase has been detected in multiple end organs of tactile sensitivity, in corpuscles of Meissner, Pacini, Herbst, and in free nerve endings, and finally at the base of auditory cells. But no transmitter of the choline group has been detected in these synapses, and it is stated by physiologists that the nerve fibers in the examples mentioned are by no means cholinergic.

It must therefore be concluded that morphological research on sensory cells, even at the submicroscopic level, do not furnish a safe and reliable base on which the satisfactory understanding of their function and activity could be founded.

Further and more extensive research, conducted in close relation with experimental work and using all the modern means of morphological investigation, is needed. There is no doubt that, in this field as in others, great hopes can be put on the abilities and perseverance of modern cytologists.

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CHAPTER 6

Connective Tissue Cells

By SYLVIA FITTON JACKSON

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I. Introduction

A. *Connective Tissue Cells and Their Environment*

The connective and skeletal tissues are concerned chiefly with the formation and maintenance of bodily structure; the ubiquitous ground substance and the predominant fibrous protein, collagen, are characteristic extracellular constituents of this otherwise heterogeneous group of tissues, which differ greatly in their physical properties and functions. The connective tissue cells and their products combine in specific ways to form various types of tissue, the nature of which depends on the relative proportions of four phases, namely, the cells; the ground substance or matrix; the fibrous components collagen, reticulin, and elastin;

and the interstitial fluid and free electrolytes. In bone and cartilage there is, in addition, the mineral phase.

The character and function of a connective tissue are determined mainly by two factors, namely the synthetic balance between the various components of the extracellular material and the precise structural relationship between the fibrous constituents and ground substance. The biosynthesis and morphogenesis of the tissues proceed in a graded series of stages each involving a variety of activities. A fundamental assumption must be made that the synthesizing cell is capable of producing all the various substances required for the formation of the extracellular material; it is not yet clear, however, whether the cell synthesizes all the products simultaneously or in regulated phases depending on the requirements of the tissue. Evidence is accumulating that the ground substance may control fibrogenesis *in vivo* and form an integral part of the fibrous material.

The cells that are most closely concerned in the formation and maintenance of the connective tissues are fibroblasts, osteoblasts, and chondroblasts associated with the connective tissue proper, bone, and cartilage, respectively. Many other types of cell may also be present. Certain aspects of the formation and deformation of connective tissues and the role of the cell in these processes have been studied now in some detail; a large body of evidence has accumulated also on the chemical characterization and structural configuration of the extracellular constituents. It will be the purpose of this chapter to examine these various facets of the connective tissue system and to construct a coherent picture of morphogenetic mechanisms; so as to keep within reasonable bounds inevitably many data will have to be omitted. The nature of the extracellular constituents will be detailed first and will be followed by a discussion on connective tissue differentiation with special reference to cellular mechanisms and the interrelation between the fibrous proteins and the ground substance. The contents of this chapter will range from the molecular level, both structural and chemical, to the final organization of the tissue since this approach may supply the wide spectrum of information required for a better understanding of the functional activities of connective tissue. It is necessary, however, to describe briefly some main features of the connective tissues before embarking on the major discussion.

B. General Characteristics of Connective Tissue

Collagen has been found in nearly every class of animal and constitutes the chief structural material of the tissues in vertebrates; the

function of collagen in invertebrates is less clear, however, and Rudall (1955) has suggested that in these two groups collagen and α-chitin bear an inverse relation to each other as the structural material. The diagnostic feature of collagen is a typical high-angle X-ray diffraction

TABLE I
DISTRIBUTION OF COLLAGEN IN PHYLA[a]

Phylum	Class	Name	Source
		Mesenchymal and mesoglcal collagens	
Vertebrata	Mammalia	Collagen	Connective tissue, cartilage, bone, hematopoietic tissue
		Vitrosin	Vitreous humor of eye
	Aves	Collagen	As for Mammalia
	Amphibia	Collagen	Similar to Mammalia
	Pisces		
	Teleostei	Ichthyocol	Skin, tendon, swim bladder
		Ichthylepidin	Scales
			Body wall of *Aquila aquila*
	Elasmobranchii	Elastoidin	Fins
Mollusca	Cephalopoda	Collagen	Connective tissue of *Loligo*
Annelida		Collagen	Body wall of *Sabella*
Hemichordata		Collagen	Skeleton of *Saccoglossus*
Echinodermata	Holothurioidea	Collagen	Body wall of *Thyone*
			Cuverian tubules of *H. forskali*
	Echinoidea	Collagen	Peristome of *Arbacia*
	Asteroidea	Collagen	Body wall of *Asterias*
Coelenterata	Anthozoa	Cornein	Axial stalks and body wall of *Alcyonium*
Porifera	Demospongiae	Spongin	Skeletal fibers of S. *graminae*
		Collagen	Body wall of *Metridium*
		Collagen	Float of *Physalia*
Onychophora		Collagen	Dermis of *Peripatus*
		Secreted collagens	
Vertebrata		Ovakeratin	Capsule of skate egg
Mollusca		Byssokeratin	Byssus threads of *Mytilus edulis*
Annelida		Collagen	Cuticle of *Lumbricus*
Brachiopoda		Collagen	Stalk of *Liugula*
Nematoda		Collagen	Cuticle of *Ascaris*

[a] Modified from Kendrew (1954) and Rudall (1955).

pattern, which is used as a hallmark for the identification of collagenous connective tissue. Table I lists the main tissues in different phyla in which collagen has been found. The heterogeneous ground substance of vertebrates differs from that of invertebrates and is characterized by the marked presence of acid mucopolysaccharides, frequently found in combination with noncollagenous protein; other substances include

glycoproteins, lipid constituents, collagen macromolecules not yet in fibrous form, and interstitial water. Little is known about the ground substance of invertebrates, but it does not appear to include much, if any, acid mucopolysaccharide.

The types of connective tissues in vertebrates vary greatly, and the structural form that obtains depends primarily on the proportions of the different extracellular constituents and the orientation of the fibrous proteins. The loose and irregular dense connective tissues form fine dividing membranes between organs and parts of organs and investing sheaths; the content of collagen in the lungs of rats is 11.3% of the total dry weight (Neuman and Logan, 1950) whereas in liver the content of the loose connective tissue is but 0.86% (Lowry *et al.*, 1941). The loose and irregular dense connective tissues facilitate movement between structures by confining each structure within its own domain; they serve as pathways for blood vessels and store fat and hence aid in the conservation of heat. On the other hand, the regular dense connective tissues, such as tendon, have an important mechanical function and are designed to withstand great stresses; the fibrous collagen content thus ranges from 76.9% (porcine) to 93% (elephant) as shown by Neuman and Logan (1950). The proportion of the various constituents of the ground substance in these tissues is not known.

Eastoe and Eastoe (1954) made a careful estimation of the amount of different substances in bone and found that the total organic material was about 20%, of which 93% was collagen, 1.2% acid mucopolysaccharide in combination with noncollagenous protein, and 5.1% a "resistant" protein. The collagen fibers of bone are heavily invested with mineral salts, but the physical characteristics of the tissue are such that they are able to provide the requisite strength without unnecessary weight. The second supporting tissue, cartilage, is markedly different; the collagen content of the tissue is only about 40% whereas the amount of acid mucopolysaccharide in combination with noncollagenous protein forms about 50% of the total dry weight. It is this latter property of cartilage that gives a characteristic metachromasia in histological preparations. These few figures of the different amounts of the main materials in diverse connective tissues illustrate the significant variations that occur and may be correlated with differences in tissue texture. Harkness (1961) has recently reviewed the biological functions of collagen.

The histological picture reflects the variations in the composition of different connective tissues; in an earlier volume Lacroix (1961) described both bone and cartilage. One of the most noticeable features is the spatial organization of the collagen fibers; for example the collagen fiber

bundles are arranged essentially in parallel array in tendon, whereas in cornea the collagen lies in layers at right angles to each other (Jakus, 1961). It is probable that the orientation of the fibers may be correlated with functional requirements, but the types of forces involved during tissue differentiation are not known; often the final pattern appears to have been influenced by the initial spatial arrangement of the cells, but Weiss (1939) has suggested that the ground substance may be concerned.

II. The Nature of the Extracellular Constituents

A. *The Ground Substance*

1. *Interstitial Fluid*

This substance bathes the whole of the extracellular compartment as well as the cells and contains plasma proteins, free electrolytes, hormones, and other circulating materials; it must carry also low molecular weight substances such as amino acids, simple sugars, peptides, and precursor and degradation products of the other moieties. It is probable that the interstitial fluid is in a colloidal suspension rather than in a free fluid state; although it is perhaps impossible to draw a clear distinction between the fluid and other components of the ground substance, its presence should not be ignored when considering fibrogenetic mechanisms.

2. *Glycoproteins*

Dische *et al.* (1958) have shown that there are small but significant amounts of glycoproteins, however, which do not contain hexuronic acid or sulfate residues, in the extracellular regions. Glucose and galactose, in combination with protein, have been identified in the vitreous body and in bone. Furthermore, a second glycoprotein has been found; it consists of galactose, mannose, hexosamine, and fucose in combination with protein. Dische *et al.* suggested that the latter material was located in the ground substance but that the former substance might be associated with collagen. Castellani *et al.* (1960) have reported the presence of sialic acid in epiphyseal cartilage of young rabbits as well as in cornea, aorta, and dental pulp; the authors concluded that sialic acid was a diffuse constituent of the ground substance. Herring and Kent (1961) have isolated from bovine cortical bone a sialoprotein with 40% carbohydrate residues including 16% of sialic acid. The functional importance of sialic acid in connective tissues is not known, but Banga and Balo (1960) have suggested that sialoprotein may be one of the stabilizing factors in collagen fibers and that it is responsible for the periodic acid-Schiff reaction in the tissues.

3. Acid Mucopolysaccharides

a. Main features. The structure of several acid mucopolysaccharides is now known in considerable detail, mainly owing to the work of Meyer and his colleagues (e.g., Meyer *et al.*, 1956; Hoffman *et al.*, 1956; Linker *et al.*, 1958). The mucopolysaccharides are all linear, unbranched polymers of disaccharide repeating units consisting of a hexosamine, either glucosamine or galactosamine, and another sugar, either a uronic acid or a galactose. The number of distinct mucopolysaccharides in connective tissues is unknown, but those that have been isolated and identified fall into two groups. Hyaluronic acid and its isomer chondroitin represent a class of nonsulfated acid mucopolysaccharide; the second group are sulfate esters and are at present represented by chondroitin sulfates A, B, and C, keratosulfate, and heparitin sulfate; the degree of sulfation varies, but usually the sulfate moiety is in equimolar proportions with hexosamine and uronic acid. Details of the main features of the mucopolysaccharides are listed in Table II.

Distribution studies have shown that different connective tissues contain a variety of acid mucopolysaccharides. Some appear to be arranged in distinctive patterns within the tissue, the type and proportion often changing with age; the exact quantity of any one mucopolysaccharide per dry weight of tissue, however, is not known in many connective tissues owing to the difficulty of quantitatively isolating these substances. The distribution of some of the acid mucopolysaccharides found in certain connective tissues in vertebrates is shown in Table III, but this list is by no means exhaustive. Mathews (1962) has recently suggested that the type of mucopolysaccharide found in a tissue may reflect an evolutionary pattern. Much of the carbohydrate moiety of the connective tissue of invertebrates appears to differ from the acid mucopolysaccharides of vertebrates, but the structural features are not clear. Polyfucose sulfate was isolated from the jelly substance of the sea urchin *Echinocardium cordatum*, in which hexosamine and uronic acid residues were completely absent (Vasseur and Immers, 1949; Vasseur, 1952). Recently Lash and Whitehouse (1960) isolated a substance from the chondroid tissue of the snail *Busycon* and identified it as a polyglucose sulfate, and Shashoua and Kwart (1959) found glucosamine, galactosamine, and ester sulfate in the hypobranchial gland.

The functional properties of the acid mucopolysaccharides are probably numerous and diverse. The importance of such substances in morphogenesis has long been debated (e.g., Baitsell, 1915), but as yet little is known in this respect; the fact that the mucopolysaccharides clearly play a prominent role in the physiology of the cell surface, however,

TABLE II

COMPARISON OF SOME PROPERTIES OF ACID MUCOPOLYSACCHARIDES ISOLATION FROM VERTEBRATE CONNECTIVE TISSUES[a]

Property	Hyaluronic acid	Chondroitin sulfates			Kerato-sulfate	Heparitin sulfate
		A	B	C		
$(\alpha)_D$	−70°	−28° to −32°	−55° to −63°	−16° to −22°	+4°	+38° to 78°
Hexosamine	Glucosamine	Galactosamine	Galactosamine	Galactosamine	Glucosamine	Glucosamine
Uronic acid	D-Glucuronic	D-Glucuronic	L-Iduronic	D-Glucuronic	(Galactose)	Glucuronic and iduronic
Position of sulfate on hexosamine		C-4	C-4	C-6	C-6	?
Hydrolysis by testicular hyaluronidase		+	−	+	−	+
Ethanol fraction precipitating Ca^{2+} salt		30–40%	18–25%	40–50%	50%	

[a] Data from Meyer *et al.* (1956), Hirano *et al.* (1961), Muir (1961), and Dorfman (1963).

TABLE III

DISTRIBUTION OF VARIOUS ACID MUCOPOLYSACCHARIDES IN DIFFERENT CONNECTIVE TISSUES

Group[a]	Tissue	Hyaluronic acid	Chondroitin	Chondroitin sulfates			Kerato-sulfate	Heparitin sulfate
				A	C	B		
I	Vitreous humor (bovine)	+						
I	Synovial fluid (bovine)	+						
II	Tracheal cartilage (human)			+	and/or +			
II	Shaft bone (ox)			+	and/or +			
III	Umbilical cord (human)	++[g]			+			
IV	Skin (porcine)	++			+	++		
IV	Ligamentum nuchae	++		+		++		
V	Tendon	+			++	++		
V	Heart valves (human)	+			++	++		
VI	Cornea (bovine)		+	+			++	
VII	Oviduct (chick)[b]	+						
VII	Aorta (human)[c]	+			+	+		+
VII	Cartilage (shark)[d]				+			
VII	Cock's comb[e]	++		+				
VII	Nucleus pulposus[f]						+	

[a] Groups I–VI: data from Meyer (1957).
[b] Schiller (1959).
[c] Kaplan and Meyer (1960).
[d] Mathews (1962).
[e] Schiller and Dorfman (1956).
[f] Gardell (1955).
[g] ++ denotes predominant, when more than one mucopolysaccharide is known to be present.

suggests a dynamic relationship with cellular function. On the other hand, it is clear that the extracellular localization of a particular mucopolysaccharide, with a specific structure and biological activity, may be correlated with certain functional properties of the tissues. Since the acid mucopolysaccharides are long-chain polymers and carry a high negative charge, they readily form reversible linkages with other compounds; they are capable of binding large amounts of salts and water and hence play an important role in diffusion mechanisms and in the regulation of water and inorganic ion metabolism. As Hamerman and Schubert (1962) have pointed out, under certain circumstances a high fixed negative charge in the immediate vicinity of a cell would help to regulate the escape of anionic metabolites. Following this line of thought, it is clear that any mucopolysaccharide chains lying within the cell might cause positively charged substances present in the cytoplasm to become bound by electro-

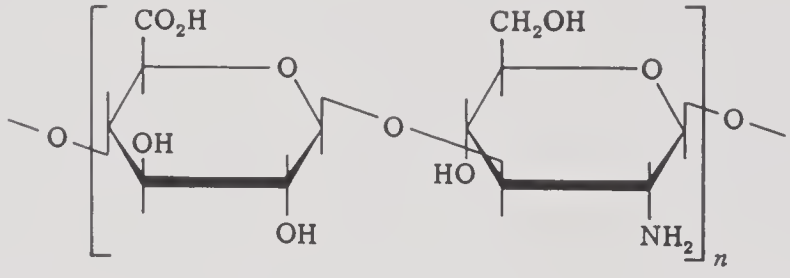


FIG. 1. Structure of hyaluronic acid.

static forces. In the extracellular regions the degree of coiling of the chains would be dependent on local ionic environment and would largely decree the physical behavior of the substance: for instance, it would control its resistance to compression, as pointed out by Fessler (1960); changes in the ionic environment could therefore alter the characteristics of the tissue quite markedly.

It should be stressed, however, that in the native state most if not all of the sulfated mucopolysaccharides are, individually, combined with noncollagenous proteins, and hence any considerations of their function should be made with this fact in mind.

b. Hyaluronic acid as a macromolecule. In 1934, Meyer and Palmer isolated an acid mucopolysaccharide from vitreous humor and subsequently a similar substance, hyaluronic acid, was extracted from bovine synovial fluid (Meyer *et al.*, 1939). Further studies on the properties of hyaluronic acid (Table II) have shown that it contains equimolar proportions of glucosamine and glucuronic acid, the composite disaccharide unit being *N*-acetylhyalobiuronic acid with the C-1 of glucosamine attached through the C-4 of glucuronic acid (Meyer, 1958) (Fig. 1).

Though hyaluronic acid is frequently found in the same tissues as sulfated mucopolysaccharides, it does not itself appear as a sulfate ester (Meyer *et al.*, 1956). Hyaluronic acid isolated from different tissues (Table III) varies in viscosity and in degree of polydispersity, but neither chemical nor enzymatic differences have been found between the isolated hyaluronates.

At one time hyaluronic acid obtained from ox synovial fluid was thought to be associated with about 20% of noncollagenous protein (Ogston and Stanier, 1952), but present evidence suggests that, though free hyaluronic acid may exist, most is bound to not more than 2% of protein (Sandson and Hamerman, 1963). Blumberg and Ogston (1957) stressed the point that protein in combination with hyaluronic acid was essential to maintain it in the state in which it occurs in synovial fluid *in vivo*.

Studies of the molecular weight of hyaluronic acid obtained from various sources show that in most samples it ranges between 1.3 and 4.5 $\times$ 10^6 (e.g., Balazs *et al.*, 1957; Laurent, 1955; Fessler, 1960), but all preparations appear to be polydispersed. Since hyaluronic acid carries a high net negative charge the degree of hydration is correspondingly high, estimates ranging from 68 to 520 ml./gm. (e.g., Fessler, 1960). With a molecular weight of 2 $\times$ 10^6 a linear chain of hyaluronic acid would consist of about 5000 repeating disaccharide units with a total length of about 5 μ. Balazs (1958) has shown, however, that the macromolecular dimensions in solution are analogous to a sphere of 3000–4000 A. diameter; thus the mucopolysaccharide chains and attendant water must be randomly coiled within this domain. Hamerman and Schubert (1962) have calculated that such spheres would interpenetrate one another at a concentration of hyaluronic acid of more than 0.1%. Hence if such a spherical configuration exists in, for instance, synovial fluid, where the concentration of hyaluronic acid is known to be as high as 0.4% (Bollet, 1956), the macromolecules must of necessity be entangled with each other. Such structural order would clearly result in a fluid of very high viscosity and would sterically obstruct the passage of certain large molecules, the concept of "excluded volume" proposed by Ogston and Phelps (1961).

Rogers (1961) has discussed the major physiological functions of hyaluronic acid macromolecules. First, it may serve to lubricate joint spaces; Ogston and Stanier (1953) pointed out that the high viscosity of hyaluronic acid and its stability under pressure applied to thin films made it an ideal lubricant. Charnley (1960), however, has challenged this concept on the grounds that the property required of a lubricant is not

so much high viscosity as "slipperiness" and that such a property may not necessarily reside in the synovial fluid but may result from an exudate from the surfaces of the articular cartilage.

Second, water retention with the resultant maintenance of turgescence, may well be a major physiological function, especially in view of the known very high hydrodynamic specific volume of hyaluronic acid macromolecules. For instance, the work of Rienits (1960) on the changes that occur in the skin of monkeys during their natural sex cycle, has indicated that there may well be a relationship between the water content and the amount of hyaluronic acid present. More direct evidence is required however to support the view that hyaluronic acid has a functional significance for water retention. The proposal of Fessler (1957), however, that the integrity of connective tissue partly depends on the location of interstitial water in "sets of sponges," of which the first is at the molecular level, is of interest; in experiments with model systems Fessler (1960) found that mixtures of water, hyaluronic acid, and collagen fibers resisted limited compression, the hyaluronic acid and water having been trapped within a network of collagen fibers. Experiments such as these may help to elucidate the function of hyaluronic acid in the tissue and suggest that relatively small concomitant changes in a water and hyaluronic acid system may lead to appreciable changes in the behavior of certain connective tissues.

Third, studies of the rate of diffusion of materials, e.g., through the skin (Duran-Reynals, 1942) and sheets of fascia (Day, 1952) demonstrated, by the use of hyaluronidase, that hyaluronic acid is a controlling factor in diffusion. This property may be due in part to the anionic character of the mucopolysaccharide; furthermore, it is reasonable to assume that the free flow of cations through the tissue is dependent on the charge profile of the macromolecules; the work of Kulonen (1953) demonstrated that in model systems cations were retained within dialysis tubing in the presence of partially purified hyaluronic acid.

Finally, Rogers (1961) has suggested that hyaluronic acid may inhibit the action of certain enzymes, as has been found with some naturally occurring macroanions (Spensley and Rogers, 1956), and furthermore, that the activity of some enzymes may be regulated by a combined system of hyaluronic acid and hyaluronidase. Such a viewpoint is of considerable importance, especially in the light of the recent finding by Bollet (1963) that hyaluronidase is present in many mammalian tissues. A combined system such as this would provide one mechanism whereby the synthetic balance of one component of connective tissue may be regulated, as discussed elsewhere in this chapter.

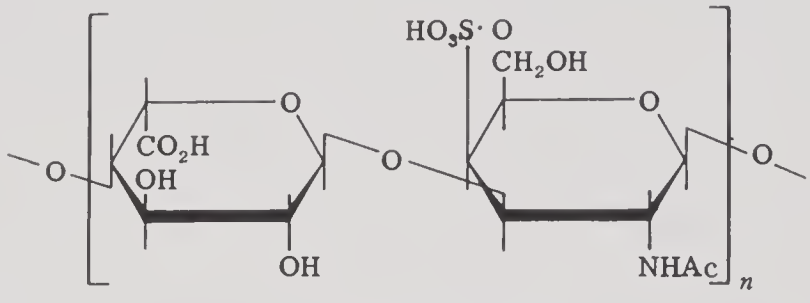
c. Chondroitin. This mucopolysaccharide was isolated by Davidson and Meyer (1954) from bovine cornea; they found that it was an isomer of hyaluronic acid, the disaccharide unit containing galactosamine and D-glucuronic acid. Since a small fraction of sulfate appeared to be associated with the polysaccharide chains, Meyer suggested that it might be a precursor of chondroitin sulfate; Adams (1959), however, has shown that chondroitin does not act as a sulfate acceptor. Thus chondroitin must be regarded as a separate mucopolysaccharide, but its functional significance is not at present known.

d. Chondroitin sulfates as macromolecules. In 1837 Müller found that the reaction of various connective tissues to autoclaving in water were very different; cartilage released a substance, chondrin, which precipitated on acidification, whereas, as had long been established, bone produced gelatin. Müller and associates were puzzled at this apparent anomaly in view of the histological belief, which was then current, that cartilage was transformed into bone. Fischer and Boedeker (1861) made detailed studies on the sulfate moiety of chondrin, and Krukenberg (1884) and Morner (1889) found that alkaline extraction methods yielded a comparatively consistent product. Subsequent work involved the identification of the type of carbohydrates present in the various preparations (e.g., Levene, 1916; Bray *et al.*, 1944; James *et al.*, 1948).

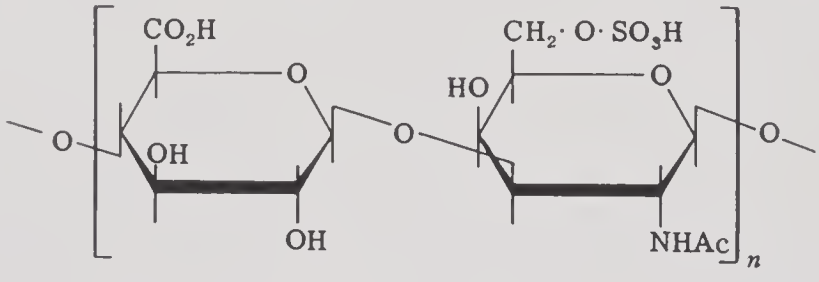
The elucidation of most of the details of the structure and properties of chondroitin sulfate, however, awaited the elegant studies of Meyer and his colleagues, who demonstrated that there are three chondroitin sulfates, A, B, and C (Meyer and Rapport, 1951; Meyer *et al.*, 1956), differing in structure and properties (Table II). Chondroitin sulfates A and C are found most frequently in cartilage whereas chondroitin sulfate B is located chiefly in skin and aorta (Table III). Chondroitin sulfates A and C have identical carbohydrate chains consisting of alternating units of galactosamine and D-glucuronic acid linked by β-glycosidic bonds (Fig. 2); on the other hand the disaccharide of chondroitin sulfate B consists of galactosamine and L-iduronic acid (Hoffman *et al.*, 1956), the repeating units thus differing from those in chondroitin sulfates A and C only with respect to the configuration of C-5 of the uronic acid moiety (Fig. 2). A sulfate group is attached by an ester link to C-4 of the galactosamine moiety in both chondroitin A and B, but to C-6 in chondroitin sulfate C (Orr, 1954; Jeanloz *et al.*, 1957; Mathews, 1958; Hoffman *et al.*, 1958). Chondroitin sulfates do not always contain one sulfate group per disaccharide unit; for instance excess sulfate has been found in shark cartilage (e.g., Mathews, 1962) whereas, in fibroblasts grown in mass tissue culture, Grossfeld *et al.* (1957) noted that the chon-

droitin sulfate produced was not fully sulfated. Recent studies by Thorpe and Dorfman (1961) have shown that the chondroitin sulfate extracted from long-bone cartilage of chick embryos is low in sulfate; these observations suggest that sulfation may occur after synthesis of the polysaccharide chain.

All three chondroitin sulfates behave as flexible linear polyelectrolytes, the degree of coiling of the macromolecule depending on the concentration and ionic strength of the surrounding milieu. After exhaustive proteolytic digestion, the chondroitin sulfate chains isolated from bovine



Chondroitin sulfate B



Chondroitin sulfate C

FIG. 2. Structure of chondroitin sulfates B and C.

nasal septa have been shown to have a molecular weight of not more than 50,000 (e.g., Mathews and Lozaityte, 1958; Mathews, 1959). If the molecular weight of each sulfated disaccharide unit is taken as 640, the number of repeating units in a chondroitin sulfate chain would be not greater than 80, giving a total chain length of 800 A.; these figures contrast sharply with those for hyaluronic acid, viz., molecular weight = 2 million, number of units = 5000, and chain length = 5 μ. It should be noted that most purified samples of chondroitin sulfate contain a small percentage of amino acid residues. Chondroitin sulfate is soluble in water, giving clear solutions up to about 10% concentration; the viscosity of the solutions increases greatly with increased protein content.

The structural differences between the disaccharide unit in chondroitin sulfate B and those in A and C are reflected in differences in

biologieal aetivity. For instance chondroitin sulfate B is resistant to testicular hyaluronidase and shows marked antithrombic activity, inhibiting blood clotting; chondroitin sulfates A and C are labile to testicular hyaluronidase digestion and are devoid of anticoagulant activity. Dorfman (1959) has pointed to the functional importance of the antithrombie aetivity of chondroitin sulfate B loealized in the aorta.

Under normal conditions chondroitin sulfates A and C do not exist in cartilage as free mucopolysaccharide but are always in combination with noncollagenous protein; in this chapter such combinants will be referred to as proteinpolysaccharides. The degree to which ehondroitin sulfate B is attached to protein is not yet known. The properties and functions of the proteinpolysaccharides will be discussed later in the chapter.

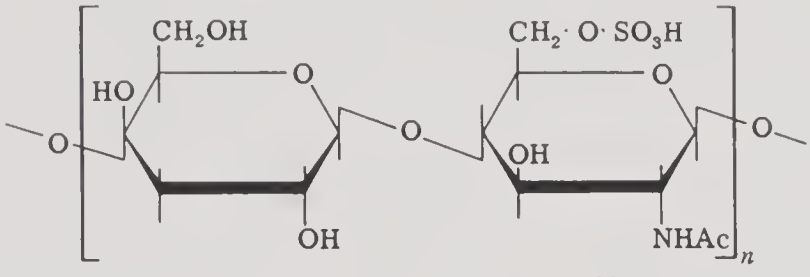


Fig. 3. Structure of keratosulfate.

e. Keratosulfate as a macromolecule. A structurally different sulfated mucopolysaccharide was isolated first from eornea (Woodin, 1952) and shown to be composed of equimolar proportions of *N*-acetylglueosamine, D-galactose, and sulfate (Meyer *et al.*, 1953), the linkage being β 1-4, β 1-3 (Hirano *et al.*, 1961). Recently 1–2% of a methylpentose has been identified in the keratosulfate of cornea and rib cartilage (Rosen *et al.*, 1960). Keratosulfate (Fig. 3) is the only known mucopolysaccharide in which a neutral sugar has been substituted for the uronic acid moiety; a correlation between this structural modifieation and a speeific functional difference, however, has not yet been demonstrated.

Keratosulfate has now been identified in a number of tissues (Table III). It appears to be combined with noncollagenous protein, but it is not yet clear whether in cartilage it is contained within the same macromolecule as chondroitin sulfate, as proposed by Partridge *et al.* (1961) or whether it is a separate entity. The funetional significance of keratosulfate is not known, but it is of interest that during human aging it has been found partially to replaee chondroitin sulfate A in costal cartilage, and, though absent in embryonic cartilage it eontributes about 50% of the total mucopolysaccharide present in that of the adult (Kaplan and Meyer, 1959).

f. Heparitin sulfate. This mucopolysaccharide has recently been isolated from certain connective tissues, e.g., aorta and amyloid (Linker *et al.*, 1958), though it was first identified as a compound in commercial heparin by Jorpes and Gardell (1948). Heparitin sulfate appears to be structurally related to heparin, since it is composed of a disaccharide repeating unit consisting of D-glucosamine and a hexuronic acid, and within the polymer chains the hexuronic moiety may be represented by both glucuronic acid and L-iduronic acid (Dorfman, 1963); alternatively there may be two structurally distinct polymers. About half the amino group is *N*-acetylated, and the other half *N*-sulfated (Ciffonelli and Dorfman, 1960) though the total sulfate content is considerably lower than that in heparin.

From studies of Hurler's syndrome, Meyer (1961) has proposed that the heparitin sulfate is a highly branched polymer. Dorfman (1963) has tentatively suggested that the mucopolysaccharide may be a metabolic intermediate in the synthesis of heparin; its physiological role is not known, nor whether the polymer is bound to noncollagenous protein.

g. Biosynthetic pathways in the formation of acid mucopolysaccharides. The sequence of mechanisms involved in the synthesis of mucopolysaccharides is not yet fully understood, but several stages have been identified. The isolation and characterization of uridine nucleotides containing various carbohydrate moieties (Cabib *et al.*, 1953; Dutton and Storey, 1954) and the demonstration of the role of uridine nucleotides in the activation of monosaccharides, as first described by Cardini *et al.* (1955) in the synthesis of sucrose, suggested that such compounds were involved in a general pattern of polysaccharide synthesis. Studies by Dorfman (1955) on the biosynthesis of hyaluronic acid have shown that glucose, without previous scission of the molecule, is a direct precursor of both the glucosamine and glucuronic acid moieties of hyaluronic acid. In further work by Markovitz *et al.* (1959) an enzyme was isolated from a strain of group A *Streptococcus* and was shown to be capable of catalyzing the synthesis of this mucopolysaccharide from uridine diphosphoglucuronic acid, incorporated into the glucuronate fraction, and from uridine diphospho-*N*-acetylglucosamine and *N*-acetylglucosamine 1-phosphate incorporated into the *N*-acetylglucosamine moiety of hyaluronic acid.

The mechanisms involved in the synthesis of polysaccharide chains composed of regularly alternating sugar units is not known, but Markovitz *et al.* (1959) have suggested that one of the steps in synthesis may involve a single enzyme with three active sites, chain formation occurring in five sequential steps without the intermediates of low molecular

weight. An alternative mechanism might involve two enzymes, one for the formation of the disaccharide uridine nucleotide and a second for polymerization of disaccharide units to polymers.

That these studies of hyaluronic acid synthesis are generally applicable to the mechanisms of acid mucopolysaccharide synthesis in connective tissues requires proof, but Roden and Dorfman (1958) have shown that glucose serves as a direct precursor of both hyaluronic acid and chondroitin sulfate B in rat skin without breakdown of the glucose chain; Lucy *et al.* (1961) in an investigation of embryonic chick cartilage have also observed that glucosamine and galactosamine appear to be formed from glucose by a direct pathway.

In recent studies of the formation of the polysaccharide chains in suspensions of cartilage cells derived from long-bone cartilage of the embryonic chick, Dorfman and Perlman (1963) using labeled precursors have found that the major pathway for the biosynthesis of the *N*-acetylgalactosamine moiety of chondroitin sulfate is from guanine-*N*-acetyl 1-phosphate to uridinediphospho-*N*-acetylgalactosamine with subsequent epimerization. In studies of cell-free systems derived from homogenates of these cell suspensions, Dorfman and Perlman found that a particulate fraction sedimentable at 105,000 *g* incorporated H^3 from *N*-acetylglucosamine 1-PO_4H^3 in a uronic acid-containing polysaccharide with subsequent epimerization; incorporation of uridine diphospho-*N*-acetylgalactosamine-H^3 indicated that the polysaccharide also contained galactosamine and though this acid mucopolysaccharide was not completely characterized it behaved like an incompletely sulfated chondroitin sulfate.

It is not yet known at which stage sulfation occurs in the synthesis of the sulfated mucopolysaccharides. Most of the available evidence supports the contention that sulfation occurs after polysaccharide chain formation. Thus in studies of the sulfation of mucopolysaccharide that may be applicable to connective tissues, Suzuki and Strominger (1960a,b) have shown that in hen oviduct the sulfate acceptor appeared to be preformed polysaccharide chains since polysaccharide synthesis did not take place. On the other hand, it must be recorded that Strominger (1962) has isolated a sulfated nucleotide.

In studies of sulfation in connective tissue, Fitton Jackson and Randall (1956a) noted in osteoblastic cultures that at first a hyaluronic acid-like material was produced; after 5 days in culture a large proportion of material was similar to chondroitin sulfate, but was low in sulfate. Grossfeld *et al.* (1957) showed that the chondroitin sulfate formed by fibroblasts from various sources grown in culture was not fully sulfated. In analyses of the chondroitin sulfate isolated from both the intra- and

extracellular zones of embryonic chick cartilage, Dorfman (1963) has found that both mucopolysaccharides are low in sulfate. Thorpe and Dorfman (1961) using a cartilage system from chick embryos, recorded incorporation of labeled sulfate in the absence of incorporation of labeled acetate, the latter being taken as a measure of polysaccharide chain synthesis; subsequently, however, they noted similar rates of incorporation of both labeled materials. As Dorfman (1963) has pointed out, these findings suggested that sulfation of polysaccharide chains may depend upon the existence, within the cell, of a pool of preformed polysaccharide chains, which subsequently become sulfated. Thus, the evidence so far available indicates that preformed polysaccharide chains undergo subsequent sulfation.

One point must be raised regarding the studies of the uptake of labeled sulfate by tissues. It is evident from the above discussion that the incorporation of S^{35}-sulfate need not necessarily be a measure of *de novo* synthesis of mucopolysaccharide chains. Furthermore, the concomitant breakdown of, for example, chondroitin sulfate in tissue slices (Coelho and Chrisman, 1960) may lead to erroneous results; the work by Suzuki and Strominger (1960c) on incorporation of sulfate reflected a statistical average, since it was found that some oligosaccharides contained more than one sulfate group per hexosamine and others none. Finally the possible presence within the cell of a pool of preformed polysaccharide chains indicate that labeled sulfate should be used only as a measure of the sulfation mechanism.

h. The turnover of acid mucopolysaccharides and their response to hormones. The rate of turnover of acid mucopolysaccharides has been studied in a few connective tissues. The work was based, at first, on measurements of the uptake of S^{35}-sulfate which was taken to represent the turnover of the sulfated mucopolysaccharide. Bostrom (1952) calculated that the half-life of chondroitin sulfate from cartilage was about 17 days, while in rat skin the turnover of S^{35}-sulfate occurred in 8–9 days (Bostrom and Gardell, 1953). In studies by Schiller *et al.* (1955, 1956), however, other portions of the mucopolysaccharide macromolecule were also labeled concomitantly, namely glucose as a precursor for the disaccharide and acetate as a precursor of the acetyl group. The results of these studies on rat and rabbit skin showed that hyaluronic acid was metabolically very active with a half-life of 2.5–4 days whereas, in agreement with Bostrom and Gardell (1953), chondroitin sulfate was found to have a slower turnover rate of about 7–10 days. All three labeled materials gave comparable rates of turnover. Recent work by Davidson *et al.* (1961) suggested that there may be an age dependence in turn-

over rates for the mucopolysaccharides. A discussion of the mechanisms involved in the breakdown of the ground substance in various connective tissues, and the mode of removal of the degradated materials, appears later in this chapter (Section V, C).

It has been recognized for many years that variatons in hormonal state affect the ground substance of connective tissues, by either an accumulation or a regression of intercellular substances (for review see Dorfman and Schiller, 1958). Zuckermann *et al.* (1938) recorded the changes in the sex skin of the rhesus monkey, and Boas and Ludwig (1950) made a careful histological study of the mechanism of estrogen inhibition of comb growth in young cockerels. In studies with S^{35}-sulfate, Layton (1951) noted that cortisone appeared to diminish the synthesis of chondroitin sulfate in cartilage, and Clarke and Umbreit (1954) found that a number of adernal glucosteroids affected the *in vitro* synthesis of chondroitin sulfate.

To analyze results in terms of quantitative changes in the acid mucopolysaccharides due to hormonal response, however, requires both precise isolation of the different acid mucopolysaccharides in the tissues and also knowledge of the normal turnover rates. In the studies by Schiller and Dorfman (1957a) on hormonal influences on skin, these facts were already known (Schiller *et al.*, 1955, 1956). They found that administration of cortisol to young rats resulted in a progressive decrease in the rate of turnover of both hyaluronic acid and chondroitin sulfate; in view of the many metabolic reactions affected by adrenal hormones, the localization of the action of excess cortisol is not known. It is probable that these effects may vary in different tissues, or even in the same tissue in different parts of the body. For example Schiller (1959) has shown that the mucopolysaccharides in cock's comb were altered by testosterone but cortisone had no effect; on the other hand, Christman *et al.* (1958) found that hydrocortisone profoundly lowered the S^{35}-sulfate values in cartilage.

In hypothydroidism, produced by feeding rats with propylthiouracil, there was a marked increase in the hyaluronic acid content of the skin (Schiller *et al.*, 1962), which was accompanied by a decrease in chondroitin sulfate; after administration of thyroxine, the amount of both substances returned close to normal. In hypophysectomized animals, however, there was a marked depletion of both hyaluronic acid and chondroitin sulfate, but after the administration of growth hormone, the normal level of chondroitin sulfate was restored, although the hyaluronic acid level varied considerably. It appears therefore that thyroxine stimulates the synthesis of both chondroitin sulfate and hyaluronic acid,

whereas growth hormone affects only the synthesis of chondroitin sulfate. The interpretation of these data, however, depends on the assumption that there is a constant pool of mucopolysaccharide in the tissues and suggests that there may be rapid changes in pool size in hypothroidism and hypophysectomized animals.

Priest (1961) has found that estradiol reduces the synthesis of sulfated mucopolysaccharides in various connective tissues of rats; he studied the simultaneous incorporation of C^{14}-glucose and S^{35}-sulfate, the synthesis being redueed by factors of 53.8% (S^{35}-sulfate) and 51.3% (C^{14}-glucose), respectively. The general importance of insulin in connective tissues and the depletion of mucopolysaceharide synthesis in, for example, diabetes (Schiller and Dorfman, 1957b) is doubtless due to the requirement for the hormone in the utilization of glucose, the lack leading to failure to synthesize the polysaccharide chains. It is thus evident that the endocrine glands play a major role in the control of the acid mucopolysaccharides.

4. Proteinpolysaccharides

a. General characteristics. Ample evidenee is now available that the sulfated acid mucopolysaccharides exist in the tissues as firmly bound complexes with noncollagenous protein. Schubert (1958) has reviewed some of the earlier work that indicated such an interrelationship. Krukenberg (1884) and Morner (1889) isolated from cartilage some carbohydrate protein products that varied in composition according to the method of isolation; Levene (1925) held that chondroitin sulfate was united with protein by an ester bond, since it was degraded by strong alkali, whereas Meyer *et al.* (1937) suggested saltlike linkages. Most of the investigations, however, were directed to the characterization of the acid mucopolysaccharide moiety.

The following discussion will center on these complexes that have been derived from eartilage: the term "protein-polysaccharide" (Gerber *et al.*, 1960) will be used to denote these substances rather than the more specific term "protein: chondroitin sulfate" since it is not yet elear whether the macromolecular configuration includes other constituents, such as sialic acid.

The proportion of protein to polysaccharide depends upon the origin of the tissue, the type of eartilage, and the age of the specimen (e.g., Malawista and Schubert, 1958; Gerber *et al.*, 1960; Johnson and Schubert, 1960). The proteinpolysaccharides exist in cartilage as salts of the dominant cation Na^+ at least 50% of this cation being bound (Schubert, 1958); there is little evidence however of a direct link with collagen,

though Partridge and Davis (1958) suggest that this might be the best way of maintaining the integrity of the tissue.

The physical properties of the proteinpolysaceharides vary widely aeeording to the method of extraction; two main types of technique have been used. The first method involved prolonged salt extraction [e.g., with 30% caleium chloride; with 30% potassium chloride and 1% potassium earbonate (Shatton and Schubert, 1954)] and subsequent purifieation; it yielded a product in which not more than 20% was protein. Further extensive purification gave about 8% protein, or ultimately only a few amino aeid residues left attaehed to the polysaeeharide moiety; occasionally these were removed by alkali (Muir, 1958). The alternative method of extraction, developed by Malawista and Sehubert (1958), involved high speed homogenization (at 45,000 rpm) in large volumes of water held below 15°C., a large part of the proteinpolysaccharides passing into solution; collagen and other extraneous matter was then removed by 2 volumes of absolute ethanol, and the proteinpolysaecharide was preeipitated from the supernatant by potassium acetate. This product was water soluble and could be purified further by reprecipitation. This basic method of extraction has led to notable advances in the study of the proteinpolysaccharides. One of the main features of products isolated in this way is a protein content higher than that obtained by salt extraetion; it is of course possible to "purify" the products until not more than, say, 8% of protein is left in eombination with polysaccharides. It is believed, however, that proteinpolysaccharides isolated from fresh tissue by means of homogenization in water more nearly represent the material as it occurs in the tissue.

The physical properties of the isolated proteinpolysaecharide are very different from those of free chondroitin sulfate. Estimates of the molecular weight of the produet vary greatly, ranging from 2×10^5 to 5×10^6; Mathews and Lozaityte (1958) have pointed out that the product can form aggregates of 50 million. The shape and size of the macromolecule will be discussed in detail in Section II, A, 4, e. The product is very viscous in solution, the viseosity depending upon protein content and state of aggregation; whether such viscosity obtains in the tissue, however, is open to question. A point of great interest, based on the high viscosity of the proteinpolysaccharides in solution, is that they are readily degraded by enzymatic action, notably by trypsin (Shatton and Sehubert, 1954; Mathews, 1956), by papain (Muir, 1958), and by streptolysin (Weissmann, 1963) as demonstrated by marked and rapid changes in viscosity (Fig. 4). Since solutions of chondroitin sulfate are not altered in this way, nor are they very viscous, it has been suggested by Muir (1958) that the protein moiety of the macromolecule is degraded and

hence that it must be concerned in the formation of the macromolecular aggregation. These *in vitro* findings clearly bear a close relation to possible degradation mechanisms in the tissues (see Section V, C).

b. The nature of the noncollagenous protein. The ready solubility of the proteinpolysaccharide immediately suggests that the protein moiety may have properties similar to those of globular proteins, but detailed information in this respect is sparse. The amino acid composition of the protein separated from the product isolated from bovine nasal

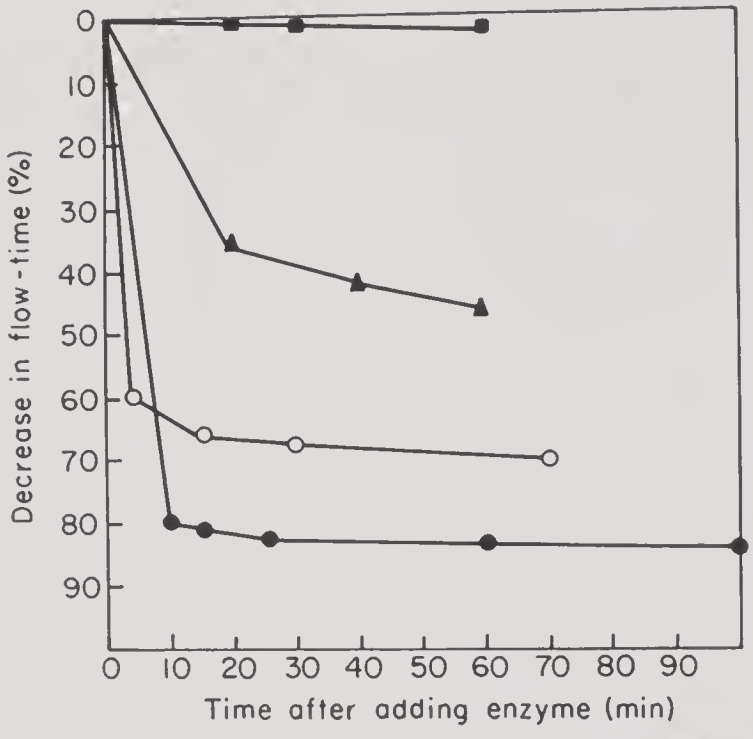


FIG. 4. Degradation of proteinpolysaccharide by papain. ● Solution (0.4%) plus crystalline papain (1 μg/ml); ○ Solution (1%) plus crude papain (10 μg/ml); ▲ Solution (0.4%) plus crude papain (10 μg/ml) dialyzed for 16 hr to remove excess of activators; ■ Solution (0.4%) plus crude papain (10 μg/ml) dialyzed for 16 hr plus *p*-chloromercuribenzoate (0.1 millimoles). Data taken from Muir (1958).

cartilage by Partridge and Davis (1958) was shown to be very different from that of collagen (Table IV), the purest preparations having a relatively low content of glycine, proline, alanine, and arginine, a high proportion of tyrosine, and no hydroxyproline. A main feature of the noncollagenous protein was the large number of acidic groups. The total nitrogen content, however, was low (about 13.5%), and only 68% of the nitrogen was recovered as amino acids. Partridge and Davis (1958) suggested furthermore that only about two-thirds of the substance was polypeptide in nature. Muir's (1958) analyses of porcine cartilage were similar, though the value for serine was double that recorded by Partridge and Davis (1958) (Table IV). Lucy *et al.* (1961) investigated the amino acid composition of noncollagenous protein from the long

bones of embryonic chicks and obtained an essentially similar pattern. The amino acid analysis of protein from bovine nasal septa by Campo and Dziewiatkowski (1962), however, indicated the presence of about 20% collagen protein.

TABLE IV

AMINO ACID COMPOSITION OF THE PROTEIN MOIETY OF BOVINE PROTEINPOLYSACCHARIDE COMPARED WITH OX BONE COLLAGEN

Amino acid	Nasal cartilage noncollagenous protein[a] (gm. amino acid/ 100 gm. dry wt.[e])	Costal cartilage protein of light fraction[b] (gm. amino acid/ 100 gm. dry wt.[d])	Ox bone collagen[c] (gm. amino acid/ 100 gm. dry wt.)
Glycine	3.64	7.38	25.3
Hydroxyproline	Nil	Nil	14.1
Aspartic acid	7.3	9.55	7.1
Threonine	3.27	4.77	2.52
Serine	2.65	7.0	4.28
Glutamic acid	12.4	16.6	11.9
Proline	7.86	7.83	14.7
Alanine	4.11	4.77	10.5
Valine	4.54	5.44	2.65
Methionine	1.22	0.77	0.80
Isoleucine	3.50	4.05	1.73
Leucine	7.73	8.61	3.93
Tyrosine	4.54	3.33	0.56
Phenylalanine	7.42	4.50	2.88
Histidine	1.83	2.22	0.96
Lysine	3.50	3.71	4.11
Arginine	2.3	5.56	9.2

[a] Partridge and Davis (1958).
[b] Campo and Dziewiatkowski (1962).
[c] Eastoe (1955).
[d] Calculated as total protein (based on 18% protein content in fraction).
[e] 68% N recovered as amino acids.

c. The nature of the linkage between noncollagenous protein and polysaccharide. Muir (1958) studied the nature of the bonds between the two moieties and found that the protein was linked to the polysaccharide through serine; she suggested that it might be combined with an acidic group of chondroitin sulfate, and furthermore, that this link responded like a weak covalent bond, which like an ester was irreversibly disrupted by dilute alkali; the bond however, resisted prolonged proteolysis. Dorfman (1962) has suggested that possibly glutamic acid, glycine, alanine, 2 uronic acid units, 1 galactosamine and 1 galactose unit may be concerned. Muir (1961) has pointed out also that the peptide fragment attached to chondroitin sulfates A and B obtained from aorta

had different amino acid patterns. Thus the form of linkage may depend upon the type of polysaccharide involved.

d. The fractionation of proteinpolysaccharides. Gerber *et al.* (1960) have shown that proteinpolysaccharide, representing about 45% of the total cartilage, isolated from bovine nasal cartilage by the extraction method of Malawista and Schubert (1958), may be separated into two major fractions by differential centrifugation. The heavier fraction, not necessarily a single component, sedimented completely at 10,000 *g* and consisted of 13% of the total dry weight of the product; this fraction was composed of roughly equal proportions of protein and polysaccharide. The light fraction comprised 75% of the total dry weight; it sedimented, in a manner that indicated a single component, with a sedimentation constant of 10.3 S and consisted of 15% protein and 85% polysaccharide. Gerber and associates pointed out that these two fractions could not be characterized as chemical individuals since preparative procedures might have caused degradation of a native complex, giving the fractions as end products. A study by Johnson and Schubert (1960) of the proteinpolysaccharide in human costal cartilage showed that the proportion of protein to polysaccharide varied rather widely in different individuals, but that the total complex represented about 35% of the dry weight of the cartilage. The product was fractionated by ultracentrifugation; 50% was separated into a light and a heavy fraction, which contained 21% and 66% noncollagenous protein, respectively. Thus it is clear from these two studies that distinct differences occur in the relative proportions of two main fractions of the proteinpolysaccharides in cartilages from different sources. Such fractions may represent distinct species of different macromolecules, or different states of aggregation of a single macromolecular species.

Campo and Dziewiatkowski (1962) examined the amino acid content of an 11.0 S fraction of proteinpolysaccharide from bovine nasal cartilage prepared according to Malawista and Schubert (1958). Since the analyses were made on the whole product, the results were expressed in grams of amino acid per 100 gm. of sample; the product was stated to contain 18% protein. The calculated values for the amino acids, based on an 18% protein content, are shown in Table IV for comparison with the figures for noncollagenous protein. It can be seen that the amino acid composition is somewhat similar to that of the separated noncollagenous protein; the amount of methionine and phenylalanine in the fractionated material appears to be lower, but the serine value is close to that found by Muir (1958).

Since it was considered essential to study the macromolecular organi-

zation of the proteinpolysaccharide as it occurred in the tissue, Fitton Jackson *et al.* (1963) prepared extracts of epiphyseal and Meckel's cartilages of embryonic and postembryonic chickens: they used the method of Malawista and Schubert (1958) except that freshly dissected cartilage was homogenized immediately in water for 1–3 minutes only, in a Potter-type homogenizer at 1800 r.p.m. After subsequent purification by alcohol:potassium acetate precipitation, the proteinpolysaccharide was separated into two fractions in 0.15 *M* KCl by centrifugation at 14,000 *g* for 10 minutes only. This treatment yielded a homogeneous supernatant fraction with a sedimentation constant of 19.9 S when extrapolated to infinite dilution. This supernatant fraction has been referred to by Fitton Jackson and co-workers as the 20 S fraction. The residue fraction was clearly of high molecular weight or highly aggregated.

These studies were consistent with the view that the 20 S fraction represented a distinct macromolecular species which was a constant feature of epiphyseal cartilage at all ages examined (13-day embryo to 70-day chicken) and was also in Meckel's cartilage. The chemical analysis of the 20 S material showed that the ratio of protein to polysaccharide in epiphyseal cartilage was about 1.5, whereas in Meckel's cartilage it was 1.1. Hydroxyproline was not found in the 20 S fraction. On the other hand the protein:polysaccharide ratio in the residue fraction was 1.75, but analyses showed that a small amount of hydroxyproline was present, implying that the residue contained some collagen (between 2 and 3%). It should be noted that no attempt has been made to extract all available proteinpolysaccharide from the tissue, in view of the possible danger of degradation; hence only the product most easily available was examined, the yield of whole proteinpolysaccharide obtained from older cartilages being less per gram of starting material than that obtained from younger cartilage. Studies by infrared spectroscopy of extracted polysaccharides, both from the whole tissue and from the proteinpolysaccharide derived therefrom, showed the polysaccharide was essentially chondroitin sulfate A and a smaller proportion of chondroitin sulfate C, chiefly present in material from older specimens. The difference between these findings and those briefly reported by Dorfman (1963) suggested that other cartilaginous regions of the long-bone rudiment from chicken embryos, e.g., the lower cone regions, may contain a greater proportion of chondroitin sulfate C.

It is important to note that Fitton Jackson *et al.* (1963) found at the beginning of the investigation that lower sedimentation constants were occasionally present, e.g., 9.5 S in some of the proteinpolysaccharide preparations. Subsequent experimentation demonstrated that lower

sedimentation values than 20 S were due to partial breakdown caused either by excessive homogenization or prolonged standing in the cold prior to centrifugation; the latter finding thus supports the observations of autolysis made by Eininder and Schubert (1950).

e. Proteinpolysaccharide as macromolecules. Several studies have been made of certain physical parameters of the proteinpolysaccharides from cartilage and have led to the proposal of various models for the macromolecular structure. Difficulties attendant on such work, however, have been the marked heterogeneity of some of the solutions and the presence of large aggregates of up to several million molecular weight (Mathews, 1956).

Blix and Snellman (1945) found that a product obtained by calcium chloride extraction of cartilage had a molecular weight of 2 to 3 $\times$ 10^5; at that time, however, this figure was thought to represent the molecular weight of a fairly pure preparation of chondroitin sulfate. As has been mentioned previously, Mathews and Dorfman (1953) first proposed that the molecular weight of chondroitin sulfate was of the order of 5 $\times$ 10^4.

A molecular weight of 1 $\times$ 10^6 was determined from investigations of the sedimentation characteristics and the viscosity of a calcium chloride extract of bovine nasal cartilage by Webber and Bailey (1956); they suggested that several polysaccharide chains of variable length were bonded together by noncollagenous protein, since tryptic digestion yielded a polydispersed product with a protein content varying from 4.5 to 10%. Muir (1956, 1958) found that the viscosity of her extracts from porcine cartilage was much reduced by papain digestion and concluded that the enzyme had destroyed a protein moiety which cemented the chains together; molecular weight was not determined. Bernadi (1957) studied the proteinpolysaccharides extracted from bovine nasal septa by 30% potassium chloride; the specimens, in 0.15 *M* phosphate buffer, gave a single sedimenting boundary in the analytic ultracentrifuge with a corrected sedimentation constant of 6.85 S, but the considerable spreading of the boundary suggested a wide polydispersity of the proteinpolysaccharide. Further studies of this product, including light scattering and viscosimetry, led Bernadi to propose that only a third of the polysaccharide was attached to protein and that the molecular weight of this product was 1.98 $\times$ 10^6 compared with 5 $\times$ 10^4 for the isolated chains of chondroitin sulfate A. By combining the data, Bernadi concluded that a spherical model for proteinpolysaccharide seemed improbable while a polydispersed system of rods of about 4000 A. in length (as deduced from the results of light scattering) was incompatible with the observed negligible value for flow birefringence. He therefore proposed a polydis-

persed system of unbranched coils in which several chains of polysaccharide were linked by protein in an end-to-end fashion.

A different molecular model however was proposed by Mathews and Lozaityte (1958). A water-extracted proteinpolysaccharide without high speed homogenization was obtained from bovine nasal septa, which on analysis gave the following values: hexosamine 24%, uronic acid 25.2%, nitrogen 6.4%, tyrosine 1.4%, and hydroxyproline 0.05%. The viscosity was given as $[\eta] = 2.5$–4.3. From detailed studies by light scattering, Mathews and Lozaityte proposed a basic molecular unit of 4×10^6 with a length of 3700 A. as best representing the macromoleecular unit. The proposed molecular model consisted of a protein core along which were distributed about 62 chains of chondroitin sulfate, each with a molecular weight of 5×10^4. From the experimental values for viscosity and molecular weight and an assumed value for partial specific volume of $\bar{v} = 0.55$, Mathews and Lozaityte calculated an axial ratio of 88, with molecular dimensions of 44.5 A. $\times$ 3910 A., pointing out that the calculated length was little affected by hydration values. They suggested that the mutual electric repulsion due to charged sulfate groups lent rigidity to the molecule, which thus behaved as a rod. Each chondroitin sulfate chain was attached by one end only to the protein core, thus allowing for an increase in lateral chain extension with decrease in ionic strength. As mentioned previously, Mathews and Lozaityte noted molecular weights as great as 50×10^6, and they suggested that such aggregates could be formed by both lateral and end-to-end association of the basic units and that additional protein might be involved in this process.

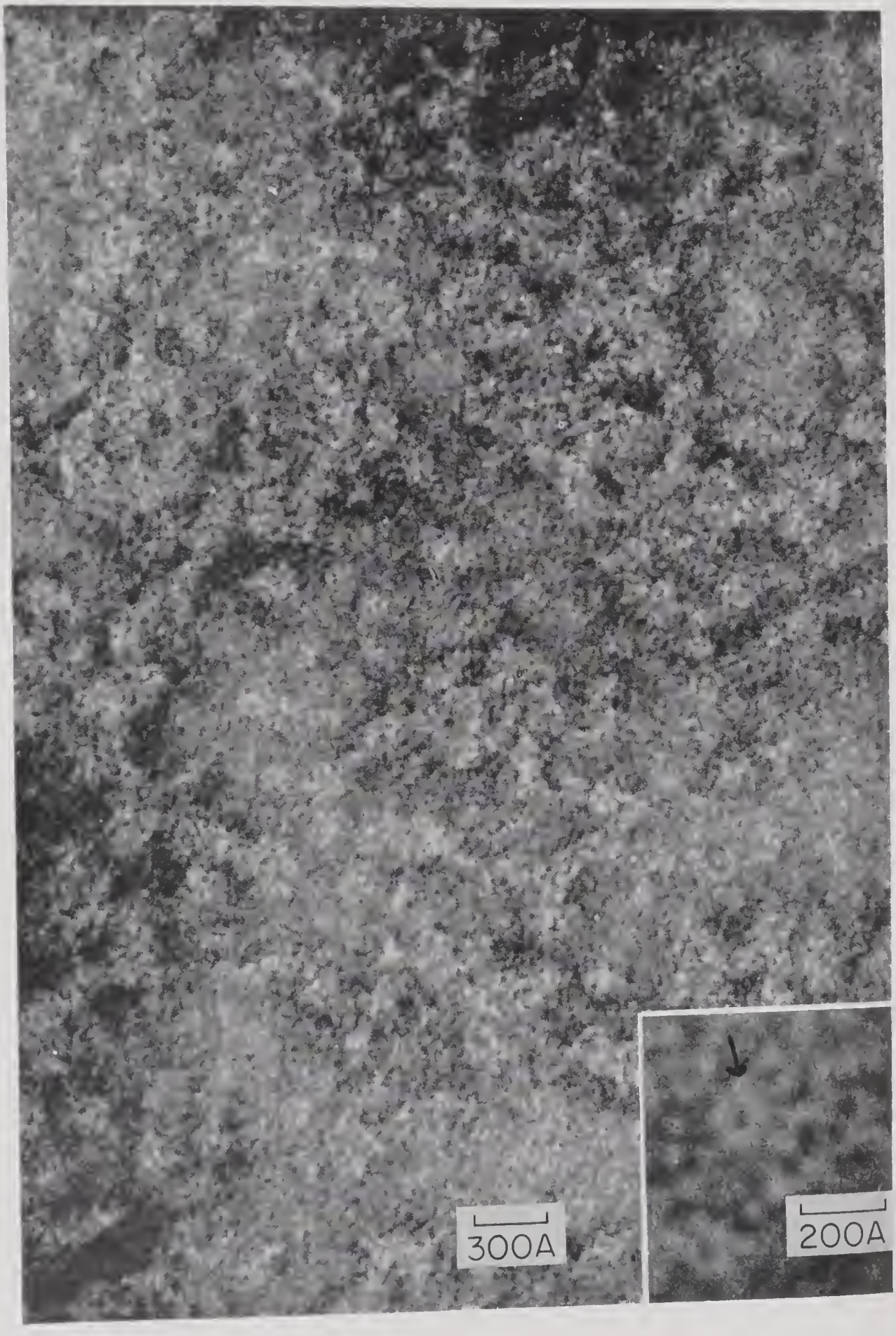
Subsequent studies by Partridge *et al.* (1961) and Partridge and Elsden (1961) were on proteinpolysaccharides obtained by water extraction of bovine nasal cartilage, two preparations of which had nitrogen contents of 8.85% and 9.85%. Their findings essentially confirmed and also considerably extended those of Mathews and Lozaityte (1958). Partridge, Davis, and Adair suggested that either the molecular weight of the proteinpolysaccharide macromoleeule in solution was between 1 and 5×10^6, or the macromolecule consisted of an aggregate of smaller units, each of 750,000. These units consisted of at least 23 chains of chondroitin sulfate, each connected to a protein core by a single linkage. The experiments yielded a molecular weight of 28,500 for each polysaccharide chain after separation from the whole proteinpolysaccharide by papain digestion. On digestion of the proteinpolysaccharide with hyaluronidase, however, it was found that chondroitin sulfate chains were split off, leaving a protein core which contained only 30% chondroitin sulfate; the osmotic molecular weight of this unit was 170,000,

thus giving a molecular weight of 120,000 for the chondroitin sulfate-free material of the core. Because of the heterogeneity of the macromolecules in solution and their tendency to form aggregates, the dimensions of the molecule were not clear.

Partridge and Elsden (1961) found that there was a carbohydrate moiety in the core material which contained both glucosamine and galactose as well as trace amounts of other neutral sugars. It was suggested, therefore, that the core might contain keratosulfate. It should be recalled, however, that recently Dorfman (1962) has suggested that both uronic acid and galactose may be concerned in the linkage of the protein moiety to chondroitin sulfate chains; hence the carbohydrate noted in the core material by Partridge and Elsden may be involved in this linkage.

A different macromolecular order has been proposed by Fitton Jackson *et al.* (1963). The 20 S supernatant fraction of proteinpolysaccharide isolated from chicken cartilage, referred to above, was also examined by hydrodynamic methods and high resolution electron microscopy. The investigations showed that all material obtained from different ages had similar properties, viz.: sedimentation constant of 19.9 S, an intrinsic viscosity $[\eta]_o$ of 0.162 ml./gm., a partial specific volume $\bar{v}$ of 0.732 gm./ml. and a protein:polysaccharide ratio of 1.5. The 20 S fraction examined by high resolution electron microscopy with the negative staining technique (Brenner and Horne, 1959; Huxley and Zubay, 1960) appeared to be composed of a mass of particulated subunits disposed in a thick mat (Fig. 5). Characteristic collagen fibrils were not observed in any of the fractions, and measurements suggested that the subunits were ellipsoids of 55 A. $\times$ 45 A. Many subunits were arranged in rings (Fig. 5) and appeared to be composed of 5–6 subunits merged together around a central core, but clear delineation of the precise organization of individual subunits within the rings was not achieved. The mean calculated value of the width of the rings was 165 A., the dimensions of the composite units being either 165 A. $\times$ 45 A. or 165 A. $\times$ 55 A. A few linear arrays of subunits were observed, but they were never composed of more than six. These observations indicated that the axial ratio of 20 S material was between 3 and 6.

The molecular weight of the 20 S proteinpolysaccharide was calculated from the experimental values for sedimentation, viscosity, and density measurements by the equation of Scheraga and Mandelkern (1953) with a β value based on the axial ratio from electron microscope observations; the molecular weight was thus $1.16 \pm 0.06 \times 10^6$. The calculated frictional ratio, $f:f_o$, was found to be 2.0, thus suggesting that


300A
200A

since the electron micrographs appeared to rule out a highly asymmetric structure, the 20 S proteinpolysaccharide was extensively hydrated. The extent of hydration was calculated from the intrinsic viscosity measurements, and a minimal axial ratio of 3, giving a minimal hydration of 4 gm. of water per gram of 20 S proteinpolysaccharide. The evidence strongly suggested that the subunits seen by electron microscopy represented only the protein content of the macromolecule, and that the polysaccharide moiety was not visualized; thus the dimensions of the composite rings measured in electron micrographs referred to the configuration of the protein within the macromolecule.

The combined physicochemical and electron microscopial results have led to the proposal of a macromolecular structure for the proteinpoly-

FIG. 6. Proposed structure for the proteinpolysaccharide.

saccharide, the model consisting of a ring of 5–6 ellipsoidal protein subunits to which a number of randomly coiled chains of chondroitin sulfate are attached around the peripheral surface, the oblate unit having a molecular weight of 1.16×10^6 and a diameter of about 220 A. depending upon the ionic environment, and hence the degree of extension of the polymer chains (Fig. 6). Since the total molecular weight of the polysaccharide in the proteinpolysaccharide is about 40% of that of the whole macromolecule, two to four chains of chondroitin sulfate would be attached per protein unit in a model composed of 5 subunits with the polysaccharide distributed equally among them.

f. The aggregation of proteinpolysaccharide macromolecules. A study of the residue fractions obtained from the proteinpolysaccharide of

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FIG. 5. Electron micrograph of the 20 S fraction of proteinpolysaccharide from chicken epiphyseal cartilage. Negatively stained with phosphotungstic acid and seen as a negative image; note the mass of particulated subunits. Inset: arrangement of the subunits in rings around the core. Total width of the unit is about 165 A.

chicken cartilage by Fitton Jackson furthermore has shown that they contain numerous long filaments (Figs. 7 and 8) which appeared to be composed of particulated subunits 45–55 A. across. The diameter of the

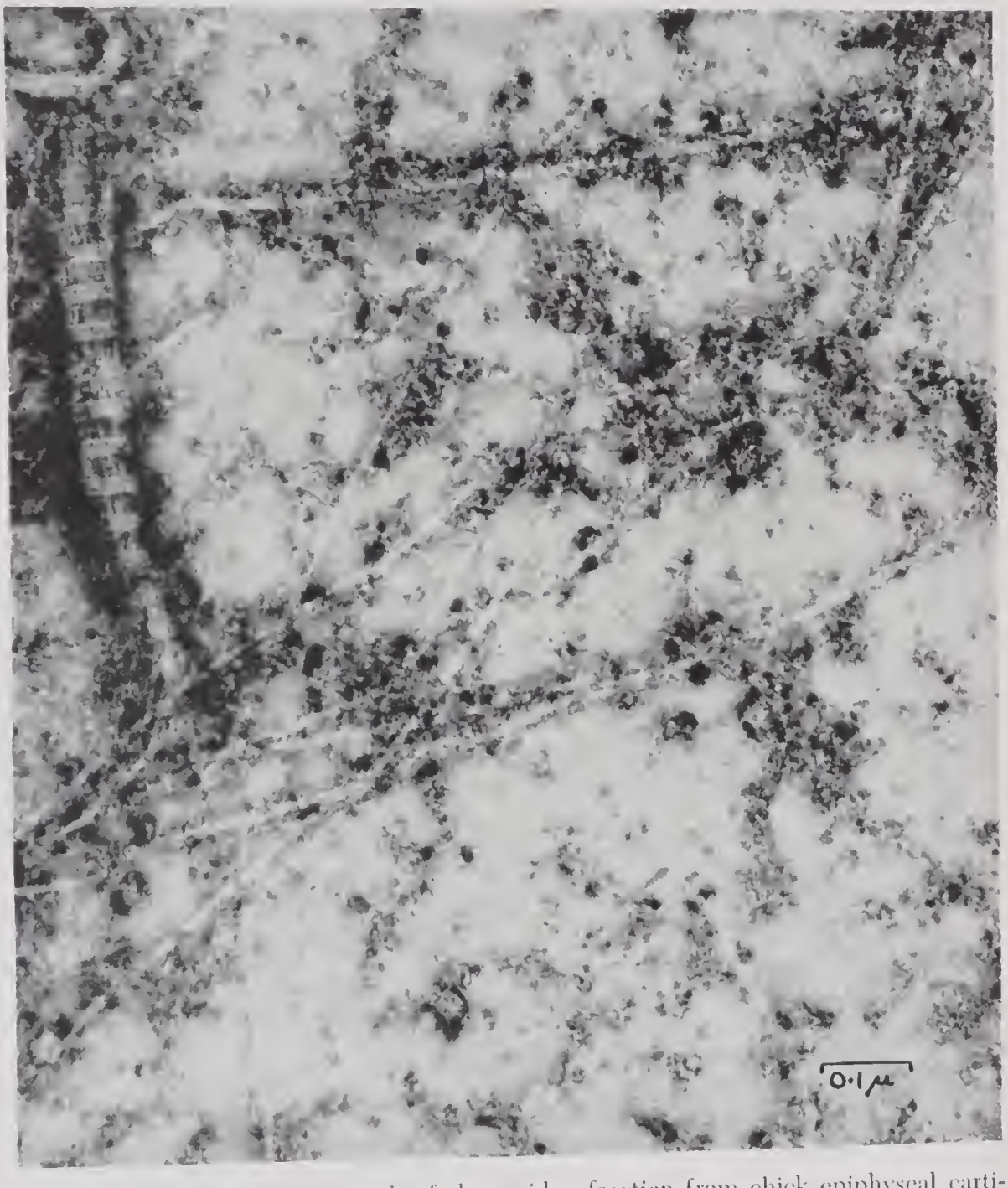


FIG. 7. Electron micrograph of the residue fraction from chick epiphyseal cartilage seen by the negative-staining technique. Much of this fraction contains long, fine filaments surrounded by a dispersion of 20 S material. Magnification: × 80,000.

filaments varied between 200–300 A. but occasionally larger filaments of filamentous ribbons were observed. Some characteristic collagen fibrils were also present, and estimates for hydroxyproline have indicated that

about 3% of the residue fraction may be collagenous; the vast bulk of this fraction, however, was composed of the particulated filaments. Hence it has been suggested that the filaments are proteinpolysaccharide in nature. Further evidence in support of this view was provided by brief digestion with papain [the enzyme that in Muir's experiments (1958) greatly reduced the viscosity of solutions of proteinpolysaccharide by release of protein], which destroyed the macromolecular order of the proteinpoly-

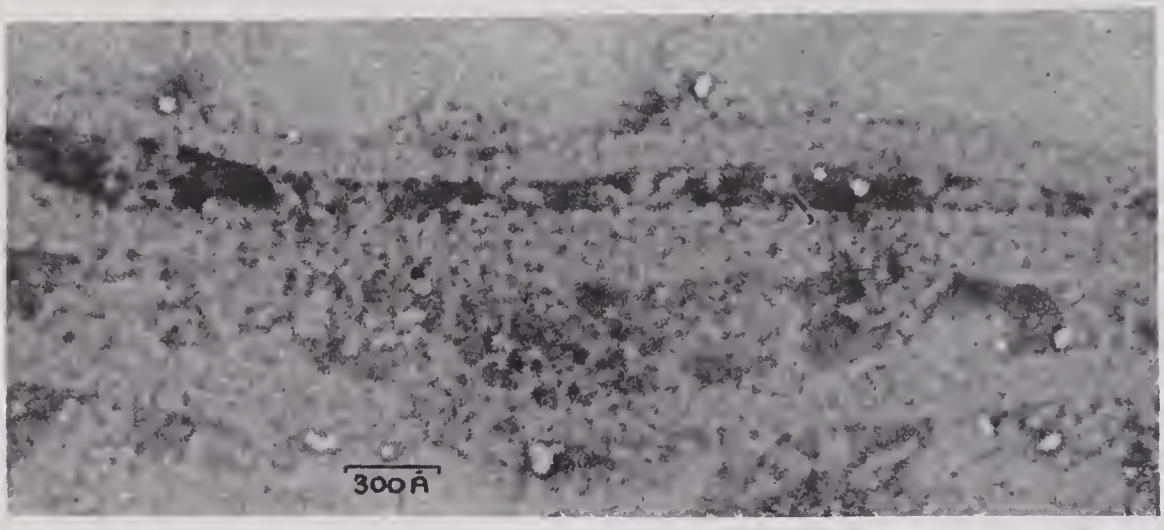
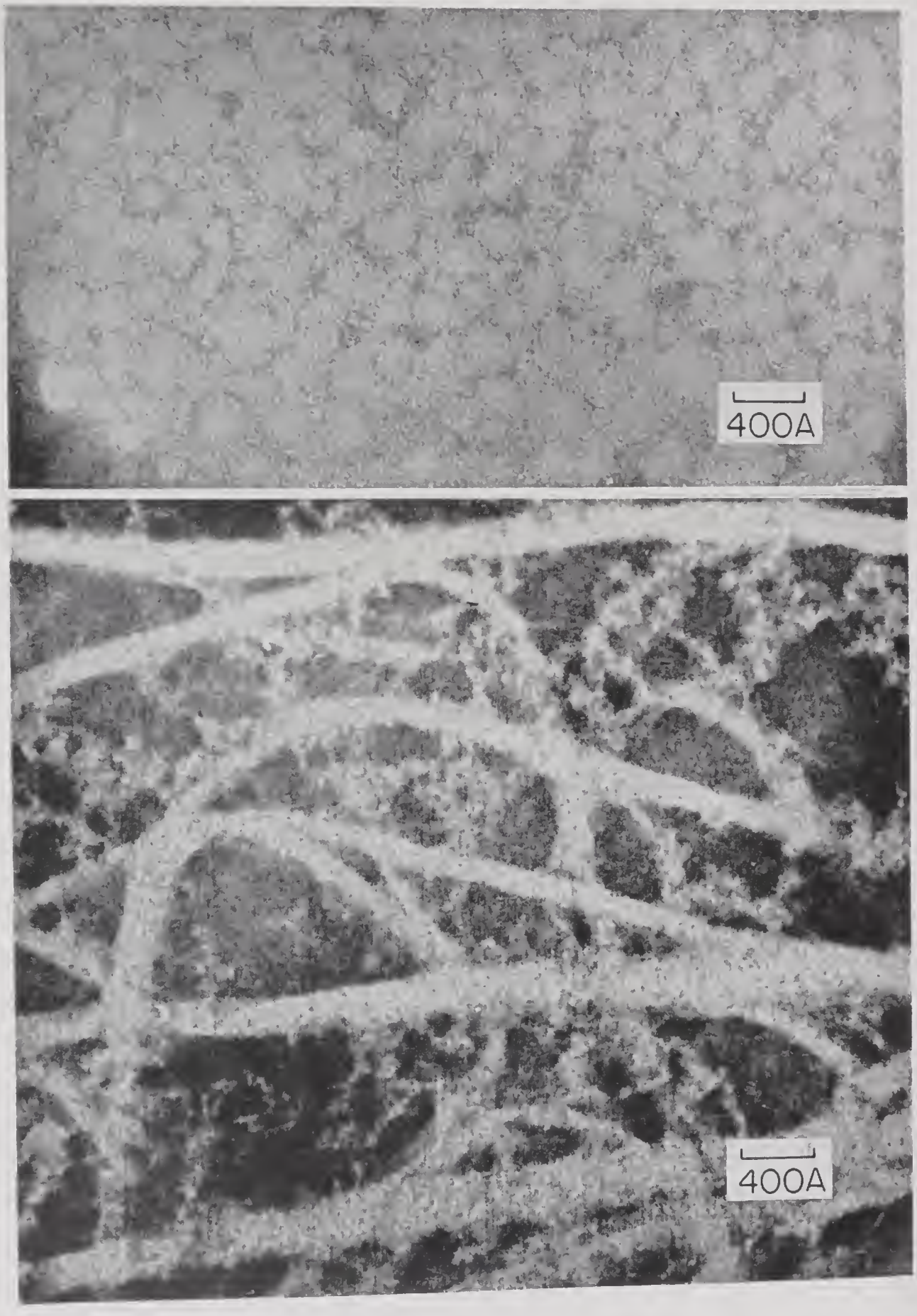


FIG. 8. Proteinpolysaccharide filaments, seen by the negative-staining technique, appear to be composed of particulate units. Magnification: × 260,000.

saccharides as seen by electron microscopy (Fig. 9); the sedimentation constant also was much diminished. On the other hand, prolonged digestion of the proteinpolysaccharide with hyaluronidase (Fig. 10) did not appear to reduce the filamentous population; Muir (1958) and Partridge *et al.* (1961) showed that this enzyme released most of the chondroitin sulfate chains but did not degrade the protein moiety.

These observations have led Fitton Jackson to suggest that the macromolecules of proteinpolysaccharide can aggregate by piling one on top of another to form filaments (Fig. 11), which are essentially cylinders of protein coated with a meshwork of chondroitin sulfate. On the other hand, the units of 1.16 million molecular weight may be formed by 5–6 subunits in linear array, which curled up to form rings when extracted from the tissue. If this were so, the filaments may be built up by lateral aggregation of 5 or 6 short strands of subunits (5 or 6 subunits per strand) aggregated either in parallel array, with slight displacement of adjacent subunits, or in helical array (Fig. 12) so as to form cylinders. Additional short strands of 5 or 6 subunits would then be added by end-to-end aggregation to form long filaments. Alternatively, filament formation may occur by the addition of individual subunits one by one


400A
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rather than by either stacking of oblate units or the aggregation of short strands.

It should be pointed out, that it is not yet clear whether the chondroitin sulfate is covalently linked to all the protein, or whether there are two or more proteins present. Experiments have shown that an individual protein cannot be easily isolated by the usual methods from proteinpolysaccharide in the fresh state, thus leaving a proteinpolysaccharide moiety of low protein content; this failure to separate out the bulk of the protein moiety may be due to its relative inaccessibility in the central region of the filaments. Some form of more severe degradation of the

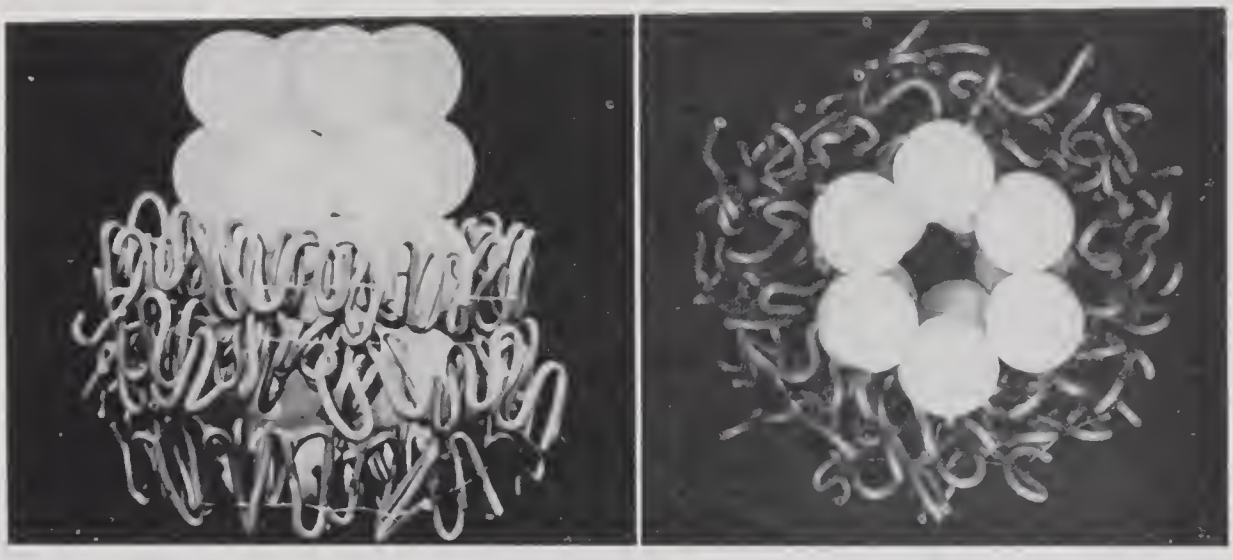

FIG. 11. Model of a proteinpolysaccharide filament to show the possible arrangement of subunits of protein surrounded by chains of chondroitin sulfate. Two views to show how the macromolecular units may be piled one on top of the other, to form into a filament.

macromolecular configuration might lead to separation of an individual protein moiety, but the author's main object is to try to demonstrate the configuration of the proteinpolysaccharide as it occurs in the tissue. If speculation may be carried one stage further, it is possible that the chondroitin sulfate is in fact only covalently linked to a small polypeptide and that such units are in turn attached to a globular protein that forms

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FIG. 9. Top: residue fraction of the proteinpolysaccharide seen after treatment with papain. The filamentous units have disappeared and the macromolecular order has been destroyed. Preparation negatively stained with phosphotungstic acid.

FIG. 10. Bottom: many filamentous units are still present in the residue fraction of proteinpolysaccharide after prolonged digestion with hyaluronidase. Negatively stained preparation.

the bulk of the filaments (Fig. 13). This hypothesis was stimulated by discussions between Dr. A. Dorfman, Dr. Partridge, and the author.

From the work discussed above, it is evident that the fractions of proteinpolysaccharides from chicken cartilage obtained by differential centrifugation merely represent different states of macromolecular aggregation. It is interesting that similar filaments to those described above

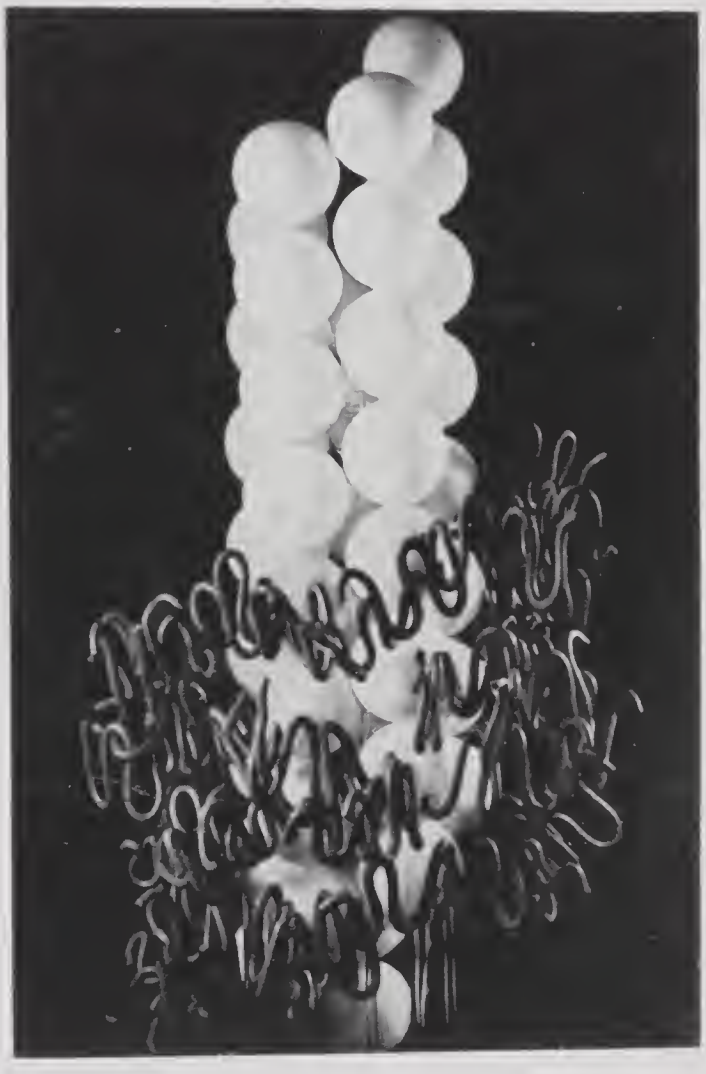


FIG. 12. An alternative model for proteinpolysaccharide filaments formed by the alignment of subunits into helical array.

are present in proteinpolysaccharide extracted from other cartilages, specimens having been kindly supplied by Dr. Schubert (human costal cartilage and bovine nasal septa), Dr. Partridge (bovine nasal septa), and Dr. Bollet (human articular cartilage). Furthermore, preliminary experiments have shown that similar structures occur in the ground substance of developing bone and dermis.

g. The synthesis of proteinpolysaccharide macromolecules. The steps involved in the synthesis of proteinpolysaccharides in cartilage are not

clear, but probably involve the production of both moieties within the same cell; this would be in line with the fundamental assumption discussed in the Introduction (Section I) that connective tissue cells are each capable of synthesizing the various products required for the formation of the extracellular material. Furthermore it is possible that other substances, e.g., sialic acid, may form part of the proteinpolysaccharide macromolecules. A recent study in the rat of the simultaneous metabolism of the protein and mucopolysaccharide moieties was made by Gerber *et al.* (1960) by means of specific labeling with DL-lysine-1-C^{14} to label the protein, and $Na_2S^{35}O_4$ as a precursor of the mucopolysaccharide portion of the macromolecule. Their results showed that there was no apparent difference between the rate of disappearance of C^{14} and

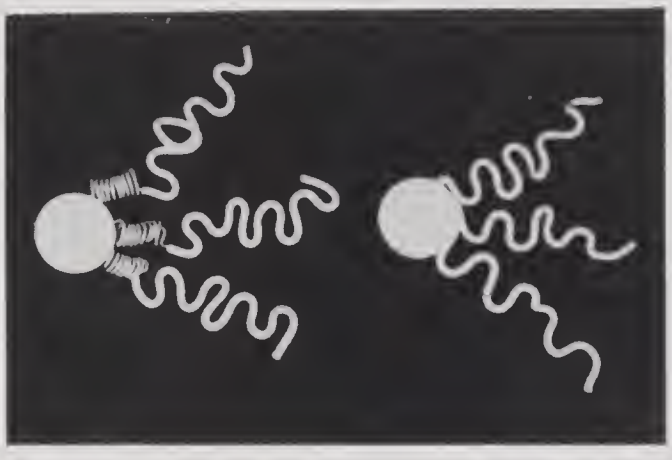


FIG. 13. Two views of the individual subunits, showing the possible relationship of the chondroitin sulfate chains to the noncollagenous protein. In the left-hand model, the mucopolysaccharide is linked to the protein subunit by a second protein.

of S^{35} and hence that their rate of turnover was the same. The log rates of turnover, however, were not linear with time, a finding that indicated that the metabolic pool of the proteinpolysaccharides was not homogeneous. Though the rate of turnover is a function of synthesis as well as degradation, the data suggested to these authors that the proteinpolysaccharide was metabolized as a unit within the cell, possibly in the molecular form of the basic unit of 4×10^6 molecular weight proposed by Mathews and Lozaityte (1958).

Studies by Campo and Dziewiatkowski (1962) of the incorporation of S^{35}-sulfate, C^{14}-L-leucine, and C^{14}-L-phenylalanine into slices of bovine costal cartilage showed that in the extracted proteinpolysaccharide the specific activity of the S^{35}-sulfate was greater than that of either C^{14}-L-leucine or C^{14}-L-phenylalanine, though the rates of incorporation were considered to be roughly parallel. In autoradiographs of the slices all three labels were predominantly localized in the chondrocytes.

Dziewiatkowski (1962) noted that 64–83% of the total S^{35}-sulfate was isolated as chondroitin sulfate, and in autoradiographs about 85% of the labeling was within the cells. These results uphold the hypothesis

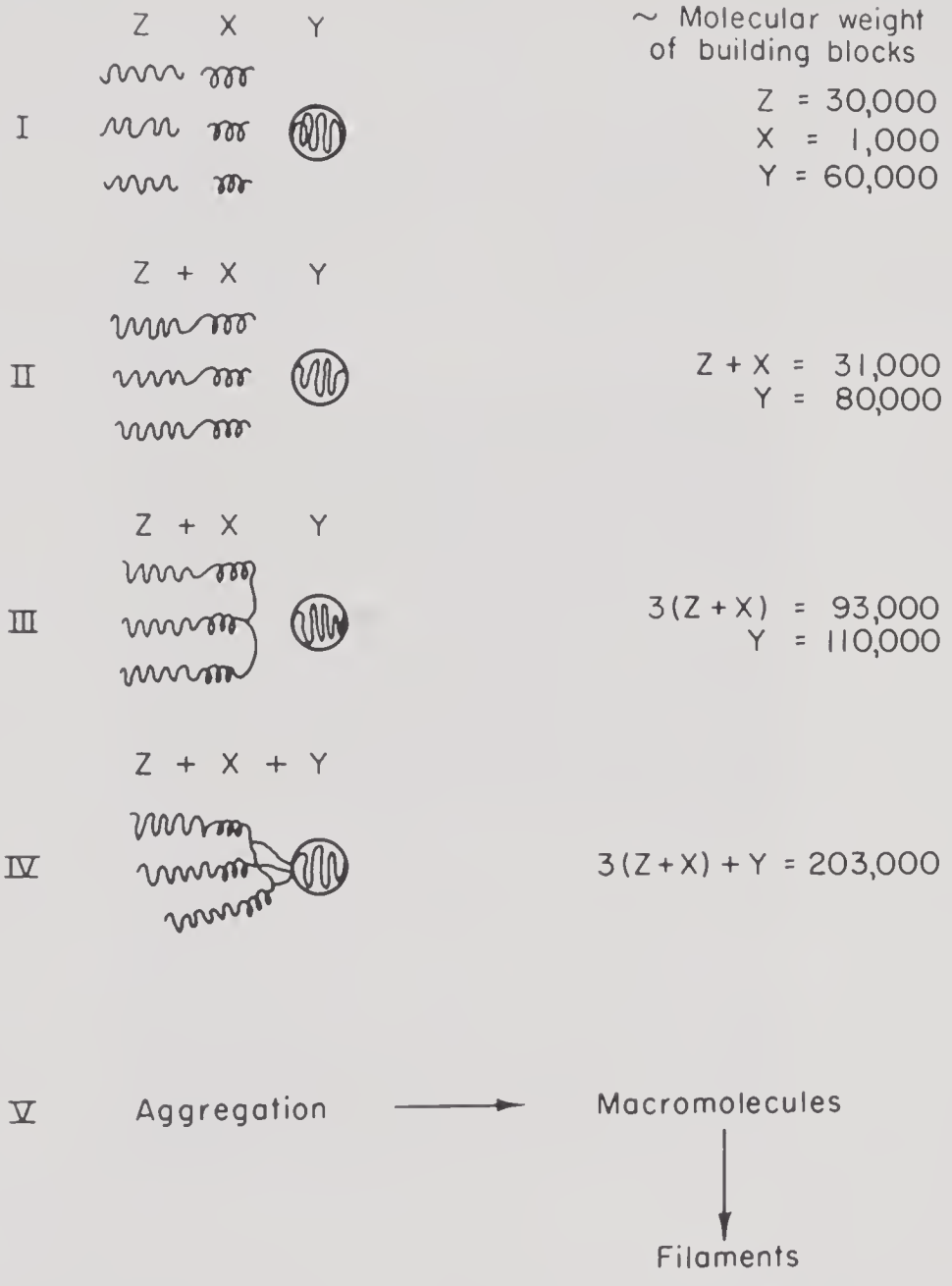


FIG. 14. Schematic diagram of the steps involved in the aggregation of the protein and mucopolysaccharide units once they have been synthesized. The scheme is hypothetical and is based on the approximate molecular weights of the various moieties. X, "linkage" protein; Y, noncollagenous protein; Z, mucopolysaccharide.

of intracellular synthesis of both the protein and mucopolysaccharide moieties. The C^{14} labels were also found to be incorporated with a collagen fraction, suggesting that the C^{14} labels represented, at least in part, collagen production.

There is no detailed experimental evidence as to the stage at which the chondroitin sulfate chains are linked to protein, and in view of the discussion in the previous section about the macromolecular configuration of the proteinpolysaccharides, it is clear that many steps may be involved. The diagram in Fig. 14 shows a possible sequence of events on a morphological level, based as far as possible on available information concerning the molecular structure, but such a scheme should be regarded with great caution until more facts have been obtained.

B. Fibrous Proteins

1. Collagen

a. Identification and general features. i. Identification. The identification of members of the collagen group of fibrous proteins in different phyla is based on a number of distinctive features; the fundamental criterion for all collagens is a characteristic wide-angle X-ray diffraction pattern which shows a strong 2.86 A. meridional spacing. Low-angle X-ray diffraction and electron microscopy of the constituent fibrils both demonstrate the presence of an axial periodicity of about 640 A. (Figs. 15 and 16) which is considered to be typical for nearly all collagen fibrils. Infrared absorption spectra provide a further simple and positive means of identification with a specific frequency at 3330 $cm.^{-1}$ of the absorption band of the NH group. The common chemical constituents of collagen include a high content of glycine amounting to nearly one-third of the total number of residues, hydroxyprolyl residues, and a higher proportion of pyrrolidine ring-containing residues than is commonly found in other proteins; there is a very low content of tyrosine and a lack of sulfur-containing amino acids.

ii. Fiber hierarchy. The bundles of collagen fibers, about 10 μ in diameter, familiar in all histological preparations, may be resolved into physically distinct structural components (Fig. 17); the individual collagen fibers within these bundles are about 0.3 μ in diameter, but in the electron microscope they are seen to be composed of many fibrils, the average diameter depending on the type of tissue from which they are derived; these fibrils in turn may be resolved into numerous protofibrils, the diameter of which is equivalent to that of the macromolecule. Collagen fibers show both form and intrinsic birefringence of visible light, the form of birefringency being positive with respect to the fiber axis; these facts show that the molecular orientation is parallel to the fiber axis. Studies of the molecular structure of collagen have led to the formulation of molecular models composed essentially of three polypep-

tide chains as minor helices hydrogen-bonded together to form a major helix.

iii. Physical properties of fibers. Two major physical properties of collagen fibers of considerable importance to the maintenance of tissue structure are their great tensile strength and their relative inextensibility.

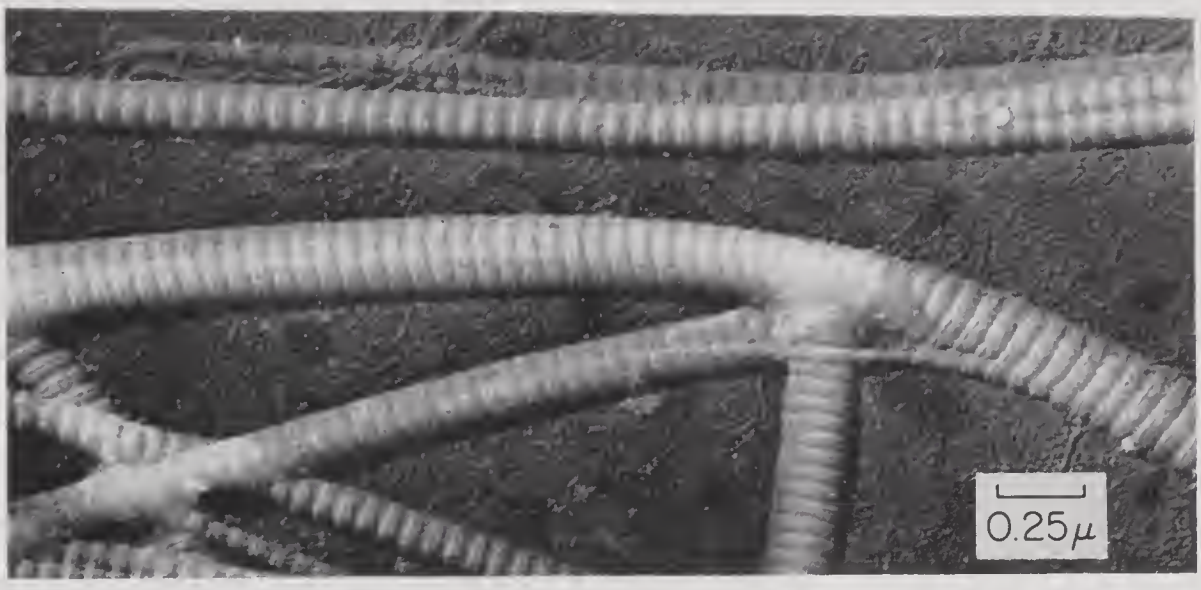


FIG. 15. Collagen fibrils seen by electron microscopy viewed after metal shadowing with gold-palladium. Note the major band region in each period. Magnification: × 40,000.

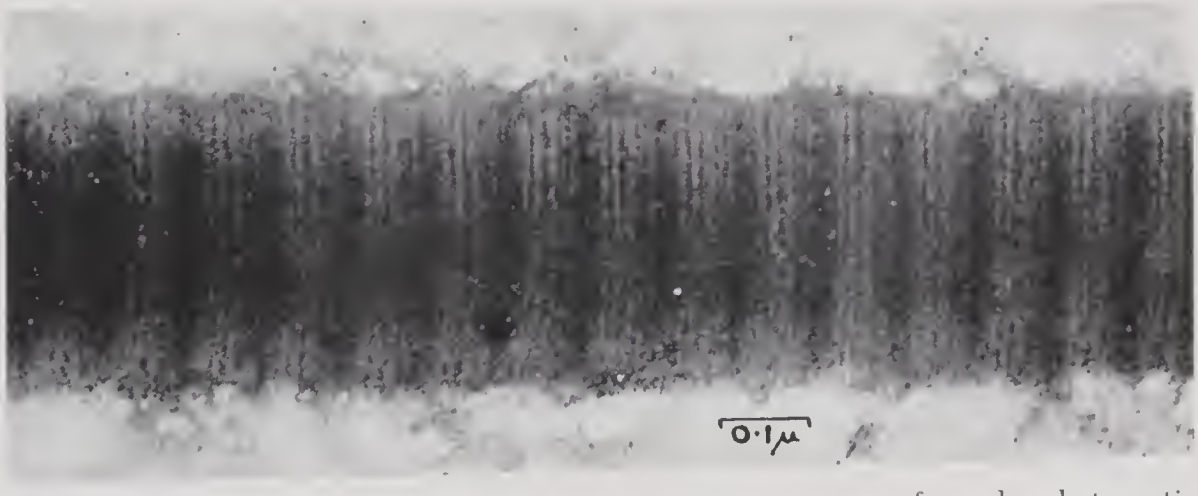


FIG. 16. A collagen fibril seen by electron microscopy after phosphotungstic staining which emphasizes a number of interbands per period. Magnification: × 120,000.

The tensile strength of most fibers has been found to be 50 kg./mm.^{2}; the strength, however, is dependent on the degree of hydration (Mitton, 1945). A 10–20% extension of the fiber can be obtained prior to rupture, and such extension is believed to occur through a straightening-out of the fibers and/or a gradual slip of one fiber relative to the next. A true

molecular extension of about 10% was obtained by Cowan *et al.* (1953) as evidenced by high-angle X-ray diffraction and was also reflected by an increase in the 640 A. axial periodicity as observed by low-angle X-ray diffraction (Cowan *et al.*, 1955).

iv. Solubility. Most of the collagen protein is insoluble unless it is subjected to heat treatment or exhaustive extraction procedures. The collagen-gelatin transition has long been known, and recently it has been shown that under certain conditions it is a reversible process (e.g., Grassmann *et al.*, 1961). A variety of soluble collagen fractions can be extracted from the tissue by quite mild methods, based on solubility at either an acid or an alkaline pH; there is also a small fraction soluble in water. A considerable proportion of the collagen is insoluble under

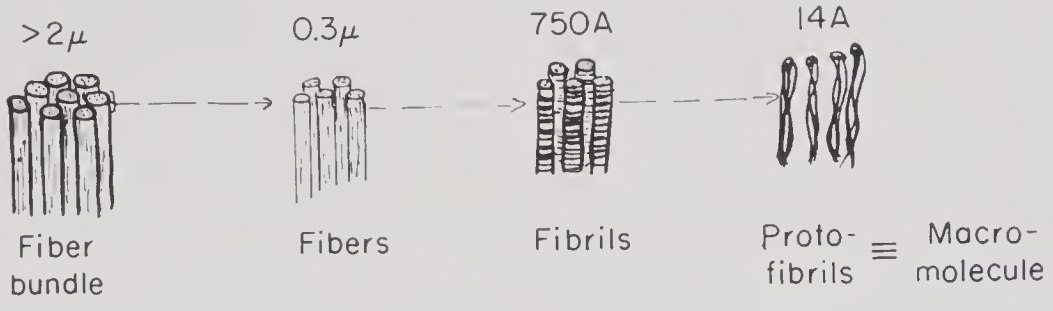


FIG. 17. Diagram illustrating the physically distinct fibrillar units of which the collagen fiber bundle is composed.

these conditions. Harkness, Marko, Muir, and Neuberger (1954) and Neuberger (1955) have found that there is a higher rate of incorporation of radioactive glycine in the alkali-soluble fraction as compared with the acid-soluble and the insoluble fractions of the tissue; these authors concluded that the alkali-soluble fraction was almost certainly the immediate precursor of the other fractions, and Orekhovitch (1952) had pointed out earlier that the acid-soluble fraction represented a young collagen. Extensive studies of various types of precipitates obtained from acid-extracted solutions of collagen led Gross *et al.* (1954) to postulate the existence of a particle "tropocollagen" roughly 2800 A. in length, which was held to represent the collagen macromolecule. Further elegant studies by Gross *et al.* (1955) demonstrated that a fraction extracted from the tissue by cold neutral salt solutions as opposed to alkaline buffers, could be precipitated as fibrils with a 640 A. periodicity by simply raising the temperature to 37°C.; such reprecipitation does not occur after acid extraction. On the basis of this work Gross *et al.* put forward the hypothesis that the neutral salt-soluble collagen was a precursor in physiological fibrogenesis.

TABLE V

AMINO ACID COMPOSITION OF VARIOUS COLLAGENS

(Residues of Amino Acids per 1000 Residues)

Amino acids	Mammalia Human Bone collagen[a]	Mammalia Human Tendon extract[a]	Mammalia Human Kidney reticulum[b]	Bovine Skin gelatin[c]	Rat Skin collagen[d]	Elasmobranchii (shark) Skin gelatin[e]	Elasmobranchii (*C. glaucus*) Elastoidin gelatin[d]	Coelenterata (*Metridium*) Body wall gelatin[d]	Echinodermata (*Thyone*) Body wall gelatin[d]	Annelida (*Lumbricus*) Cuticle gelatin[f]	Nematoda (*Ascaris*) Cuticle gelatin[g]
Glycine	319	324	309	334	327	333	319	308	306	324	286
Proline	123.4	126.4	97.2	129	117	113.4	119	63	109	13	280
Hydroxyproline	100.2	92.1	107.7	92	100	78.5	72	49	60	165	24
Imino acid total	**223.6**	**218.5**	**204.9**	**221**	**217**	**191.9**	**191**	**112**	**169**	**178**	**304**
Aspartic acid	47	48.4	52.9	48	47	42.6	45	81	62	56	76
Glutamic acid	72.2	72.3	76.7	72	74	65.8	80	95	110	81	101
Acidic amino acid total	**119.2**	**120.7**	**129.6**	**120**	**121**	**108.4**	**125**	**176**	**172**	**137**	**177**
Amide N	37.3	44	43	41	51	29.4	32	71	75	75	44
Net acidic groups	**81.9**	**76.7**	**86.6**	**79**	**70**	**79.0**	**93**	**105**	**97**	**40**	**133**
Arginine	47.1	49	45.3	48	49	50.3	50	57	54	21	42
Histidine	5.8	5.4	5.3	4.6	5.1	7.4	4.4	5.1	2.8	0	8.4
Lysine	28	21.6	21.6	25	29	24.3	24	27	7.5	15	37
Hydroxylysine	3.5	8.9	12.2	6.8	5.7	4.7	7.0	25	11	0	0
Basic amino acid total	**84.4**	**84.9**	**84.4**	**84.4**	**88.8**	**86.7**	**85.4**	**114.1**	**75.3**	**36**	**87.4**

TABLE V (*Continued*)

Amino acids	Mammalia Human Bone collagen[a]	Mammalia Human Tendon extract[a]	Mammalia Human Kidney reticulum[b]	Bovine Skin gelatin[c]	Rat Skin collagen[d]	Elasmobranchii (shark) Skin gelatin[c]	Elasmobranchii (*C. glaucus*) Elastoidin gelatin[d]	Coelenterata (*Metridium*) Body wall gelatin[d]	Echinodermata (*Thyone*) Body wall gelatin[d]	Annelida (*Lumbricus*) Cuticle gelatin[f]	Nematoda (*Ascaris*) Cuticle gelatin[g]
Alanine	113.5	110.7	96.5	105	106	119	123	113	71	103	69
Phenylalanine	13.9	14.2	18.1	13	13	13.9	15	12	8.9	5.2	7.3
Leucine	25.5	26	35.8	25	25	23.9	19	37	22	29	18
Isoleucine	13.3	11.1	18	11	10	19.4	18	23	13	15	14
Valine	23.6	25.4	26.8	19	22	21.9	17	34	30	17	13
Methionine	5.3	5.7	8.6	6.6	6.3	10.0	13	8.8	2.2	0	12
Proline	123.4	126.4	97.2	129	117	113.4	119	63	109	13	280
Nonpolar amino acid total	**318.5**	**319.5**	**301.0**	**308.6**	**299.3**	**321.5**	**324**	**290.8**	**256.1**	**182.2**	**413.3**
Serine	35.9	36.9	42.8	38	41	44.5	42	54	54	105	23
Threonine	18.4	18.5	21.9	17	20	25.8	23	39	39	52	18
Tyrosine	4.5	3.6	3	4.7	3.2	1.4	9.2	7.9	7.9	0	1.2
Hydroxyproline	100.2	92.1	107.7	92	100	78.5	72	49	60	165	24
Hydroxylysine	3.5	8.9	12.2	6.8	5.7	4.7	7.0	25	11	0	0
Hydroxy amino acid total	**162.5**	**160.0**	**187.6**	**158.5**	**169.9**	**154.9**	**153.2**	**174.9**	**171.9**	**322**	**66.2**

[a] Eastoe (1955).
[b] Windrum *et al.* (1955).
[c] Piez and Likins (1960).
[d] Piez and Gross (1959).
[e] Eastoe (1957).
[f] Watson (1958).
[g] Watson and Silvester (1959).

b. Chemical composition. i. Amino acid pattern. The amino acid composition of collagen has been studied extensively, and detailed analyses are now available for 32 different vertebrate and 10 invertebrate collagens, mainly owing to the work of Eastoe and Leach (1958), Watson and Silvester (1959), Gross and Piez (1960), Piez and Likins (1960), and Piez and Gross (1960). Some representative analyses for both vertebrate and invertebrate collagens are given in Table V; the amino acids are arranged in groups, the totals of which are shown, as an aid for discussion on their structural significance.

The outstanding feature of the amino acid composition of all collagens so far analyzed is that a minimum of 300 residues per 1000 residues[1] are glycine (Fig. 18) with the one exception of *Ascaris* cuticle, in

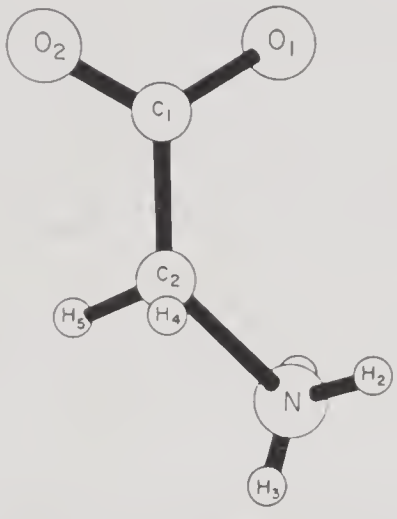


FIG. 18. The structure of glycine. After Harrington and von Hippel (1961a).

which there are 286 residues (Watson and Silvester, 1959). Two features common to all collagens are first, the small proportion of tyrosine and the absence of tryptophan, hence the minimal ultraviolet absorption of collagen at 280 mμ; and, secondly, the low content of sulfur-containing amino acids. A further characteristic is the occurrence of both hydroxyproline (Fig. 19), and, to a lesser degree, hydroxylysine; the latter is believed to be peculiar to collagen. Furthermore, there is usually a high content of pyrrolidine ring-containing residues.

The amount of amino acid other than glycine varies widely in collagens from invertebrates; in those from vertebrates also some differences are apparent, in both the distribution of amino acids in the same tissue

[1] Eastoe and Leach (1958) have pointed out that the use of the expression "residues of amino acid per 1000 total residues" permits direct comparison of data and is more useful when considering amino acid sequence and three-dimensional structure.

in different species (e.g., skin in various fish) and the distribution in different tissues of the same species. Gross (1963) has made a detailed comparative study of the available data.

In vertebrate collagen there is a signifieant content of hydroxyproline; the two amino acids, proline (Fig. 19) and hydroxyproline together account for about two-ninths, while alanine usually contributes about one-ninth, of the total residues; thus glycine, proline, hydroxyproline, and alanine constitute about two-thirds of the available residues. This is not neeessarily so in collagen from invertebrates, however; hydroxyproline varies widely in the different samples examined, ranging from only 24 residues per 1000 in *Ascaris* euticle to 165 residues per 1000 in the *Lumbricus* eutiele, while proline shows a reverse trend with 280 and 13 residues, respectively (Watson and Silvester, 1959). Hydroxyproline is absent in the cuticle of both *Lumbricus* and *Ascaris*, but is prominent

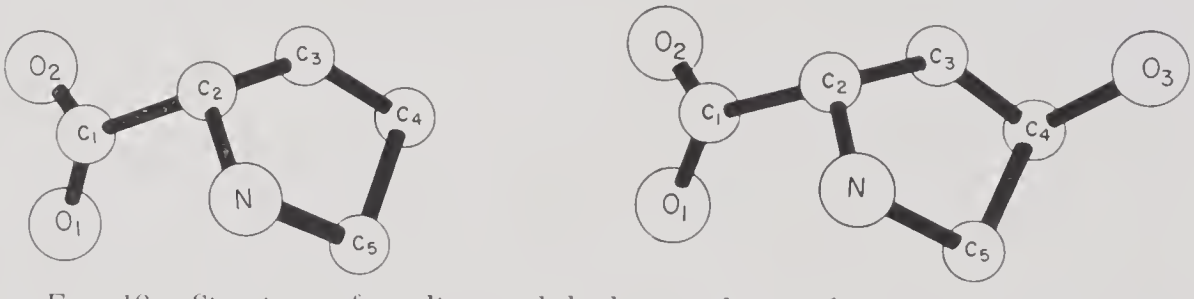


FIG. 19. Structure of proline and hydroxyproline. After Harrington and von Hippel (1961a).

in some of the other invertebrate collagens, e.g., float of *Physalia* (Piez and Gross, 1959). Four invertebrate collagens give values for alanine similar to those found in vertebrates, e.g., body wall of *Metridium*, but this level is reduced eonsiderably in other eollagens from invertebrates, e.g., body wall of *Thyone*.

The total number of basie amino acids are closely similar in eollagens from both vertebrates and invertebrates, but with the exeeption of arginine, the range of individual values is greater in the invertebrates. The amount of acidie amino acids is similar in all vertebrate collagens, the combined total being a little over one-ninth of available residues; there is a certain range of values in the invertebrates, and the total is consistently higher than that in vertebrates. Furthermore the amount of amide nitrogen is greater in most samples of invertebrate collagen than in collagen from vertebrates.

The two cuticle collagens from *Lumbricus* and *Ascaris* elearly stand apart from all others and furthermore are grossly dissimilar from eaeh other. The marked differences in composition may be due to environmental factors, the *Ascaris* cutiele having to withstand a strongly acidic

and enzymatic milieu. It is of interest that the collagen fibrils from both cuticles do not appear to have an axial periodic structure (Reed and Rudall, 1948; Watson and Silvester, 1959). These two collagens will be excluded from further discussion.

The fact that nearly one-third of the total residues of collagen are glycine shows that the amino acid is fundamental to the structure of the collagen molecule. It is of interest that the level of total imino acids in mammalian collagen is very consistent, but that in fish collagens is somewhat lower, largely owing to a smaller content of hydroxyproline; these findings led Eastoe (1955) to suggest that an environmental factor may be involved, even though his analysis of skin collagen from the whale showed similar total figures for imino acids to those in other mammalia. The imino acid totals in invertebrates however is lower than that in fish. Harrington (1958) has pointed out that the insertion of proline into a polypeptide chain would effect chain direction, and that the pyrrolidine rings might be an important factor in maintaining molecular configuration; hydroxyproline also may play a significant role in interchain or intermolecular bonding. The totals of hydroxyamino acids show a consistent level in all collagen with the exception of spongin A, where it is significantly higher (Piez and Gross, 1959); these hydroxyamino acids are doubtlessly involved in hydrogen bonding between chains, and possibly between molecules. The totals of the nonpolar amino acids are consistent in vertebrates but show a lower level and a greater range of variation in the invertebrates. Bear (1952) pointed out that it was probably the nonpolar amino acids that contributed significantly to the characteristic X-ray diffraction pattern of collagen. The total of basic amino acids is very consistent in vertebrates, as also are the net acidic groups; the basic groups usually slightly exceed the net total for the acidic groups. In collagens from invertebrates, the basic amino acids and net acidic groups vary greatly when different phyla are compared (e.g., Table V), but the respective totals within any one phylum are similar. The basic and acid groups may be of especial significance in fibrogenetic mechanisms.

It must be stressed that while the amino acid composition of different collagens may vary in detail, they all have a distinctive pattern and all display the characteristic high angle X-ray fiber diagram. The variations in the amino acid composition support the contention that the typical X-ray pattern is obtained from a "crystalline" part of the collagen fiber only, while a smaller proportion of the fiber is amorphous in the sense of not contributing to the fiber pattern. In this amorphous region of the fiber considerable replacement of certain residues by others may occur

within limits so far undetermined, but these alterations would not be reflected in the high-angle X-ray fiber diagram.

ii. Carbohydrate content. Schmitt *et al.* (1955) suggested that about 0.5% carbohydrate was intimately associated with purified vertebrate collagen. Most of the carbohydrate was identified as galactose and glucose, but there were also trace amounts of mannose; the hexosamine content was about 0.1%. On the other hand, the content of tightly bound carbohydrate in invertebrate collagen is comparatively large, ranging between 5% and 15% (Watson, 1958; Gross *et al.*, 1958; Watson and Silvester, 1959). Glucose, galactose, mannose, fucose, and ribose have been identified and occur in varying amounts depending upon the source of material; the amino sugar content is also noticeably higher than that in vertebrates, both galactosamine and glucosamine being present.

c. Molecular configuration. The characteristic wide-angle X-ray diffraction diagram which represents the configuration and packing of polypeptide chains in collagen has been known for about forty years and was used with great effect by, e.g., Marks *et al.* (1949), in the identification of the presence of collagen in different phyla. Astbury (1933) pointed out that the diffracting pattern obtained from collagen suggested that the configuration of the polypeptide chain was unlike that of either α or β protein, and in 1947 Astbury proposed that collagen should be classified as a separate group from that of the keratin-myosin-epidermal-fibrinogen group of fibrous proteins. Recently, however, Rudall (1962) has queried this classification and suggested that basically all the fibrous proteins belong to the same family in the sense that the main diffracting unit of the polypeptide chains, common to all fibrous proteins, is derived from 50% or more of small amino acid residues. The earlier work on collagen structure was reviewed by Bear (1952) and the more recent work by Harrington and von Hippel (1961a).

The present proposals for the structure of collagen have been due, in the main, to the many and detailed studies made by Bear (e.g., 1952, 1956), Ramachandran and colleagues (e.g., Ramachandran and Kartha, 1954, 1955; Ramachandran *et al.*, 1962), Randall and colleagues (e.g., Randall, 1954; Cowan *et al.*, 1953, 1955; Bradbury *et al.*, 1958), and Rich and Crick (1955, 1958, 1961). Since an understanding of the molecular configuration is a prerequisite for studies of fibrogenesis, the main details will be outlined briefly.

A notable and characteristic feature of the collagen wide-angle X-ray diffraction pattern is an axial periodicity of 2.86 A., which represents

the projected length between adjacent residues. A prominent equatorial reflection of 10.4–15 A., the spacing depending on the degree of hydration, indicates that the diameter of the fundamental lateral unit (protofibril in Bear's terminology) is of this order. There is now some general agreement on the broad outlines of the configuration of the collagen molecule; the development of the structural models was greatly aided by improved X-ray diffraction diagrams obtained by North (Cowan *et al.*, 1953), which gave a sharper pattern and contained many more discrete reflections than previously (Fig. 20). The diagram indicated the possibility of a helical structure for collagen.

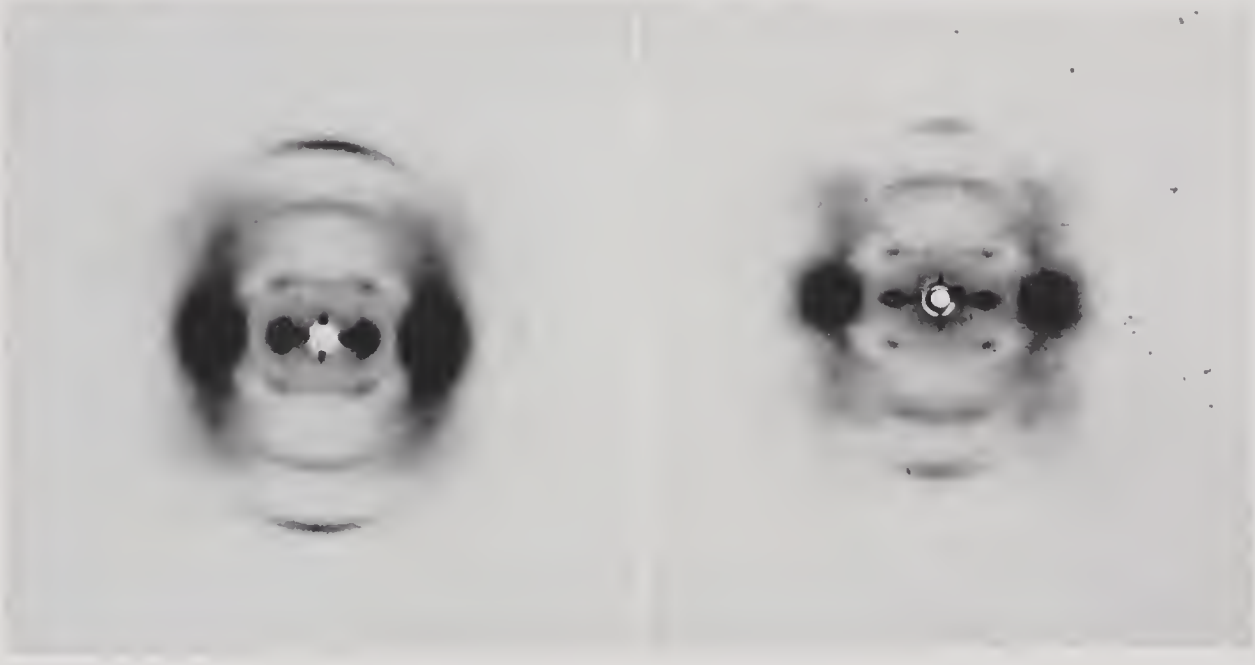


FIG. 20. X-ray diffraction patterns of collagen from rat tail tendon: left, normal; right, stretched 8%. Courtesy of Cowan *et al.* (1953).

In 1954 Ramachandran and Kartha proposed a three-chain helical structure, a proposal which has since been shown to have been along the right lines. Rich and Crick (1955) and Cowan *et al.* (1955) proposed that two alternative but closely related helical models of the collagen molecule could be constructed which agreed essentially with the X-ray, infrared, chemical, and physicochemical data.

The models consisted of three separate polypeptide chains each individual chain being coiled into a threefold left-handed helix, which gave three amino acid residues per turn, and a 9.4 A. rise along the fiber axis. This formed the polypeptide backbone of the structure and was similar to that of poly-L-proline (Cowan and McGavin, 1955) and poly-L-glycine (Crick and Rich, 1955). Three such chains were arranged about 5 A. apart, with their axes parallel so as to form a compact unit,

and related by a threefold screw axis running up the center of the group. In this arrangement every third amino acid residue of each chain would be in an identical environment, and every third residue must be glycine. The chains were stabilized by lateral interchain hydrogen bonds made between every third amino acid residue of adjacent chains at the same level, and the bonding could occur between a glycine NH of one chain and a carboxyl group of an adjacent chain. There were two alternative methods of bonding: in structure I (Rich and Crick, 1955, Terminology) the NH group pointed anticlockwise, and in structure II clockwise, when viewed from the carboxyl ends of the chain. In collagen II the OH group of hydroxyproline would stick out radially from each unit of three chains and thus could form a hydrogen bond with a neighboring molecule; in collagen I the OH group lies within the unit of three chains and thus could be used in intrachain bonding. The three chains did not lie parallel to the general fiber axis, however, but wound round one another to form a gradual right-handed helix, of large pitch, around a common axis.

For a model of equivalent polypeptide chains, collagen II was stereochemically more satisfactory since all the van der Waals contacts and bond angles were reasonable, whereas in the collagen I model, some of the contacts were short and others long and the bond angles were distorted. The model proposed by Ramachandran and Kartha (1955) differed in the way in which the chains were packed together; it employed two interchain peptide hydrogen bonds for every three-residue repeat. Subsequent physicochemical and infrared studies by Burge and Hynes (1959) and Bradbury *et al.* (1958) favored the collagen II structure, and further analysis by Rich and Crick (1958, 1961) emphasized the stereochemical correctness of collagen II (Fig. 21). Beer *et al.* (1958), however, favored the "double-bonded" structure of Ramachandran, and in further work Ramachandran *et al.* (1962) have stated that the double-bonded model is more satisfactory. Harrington and von Hippel (1961b) have suggested that water is concerned in the stabilization of the collagen molecule by means of hydrogen-bonding of adjacent peptide carboxyl groups in the same polypeptide chain.

As pointed out by Rich and Crick (1961) the collagen molecular model forms an elongated cylinder; since glycine has only a hydrogen atom in place of side chains of other amino acids, the repeat period of glycine as every third residue along each chain forms a deep helical grove (Fig. 21). A helical ridge is formed by the presence of amino acids other than proline or hydroxyproline, with long side chains. Thus the ridge of one molecule tends to fit into the grove of an adjacent molecule

and may be an important factor in stabilizing the molecular order in the intact collagen fibril.

d. Amino acid sequence in peptides. Studies of the amino acid sequence in peptides derived from collagen have lent strong support for the formulation of models of the configuration of the collagen mole-

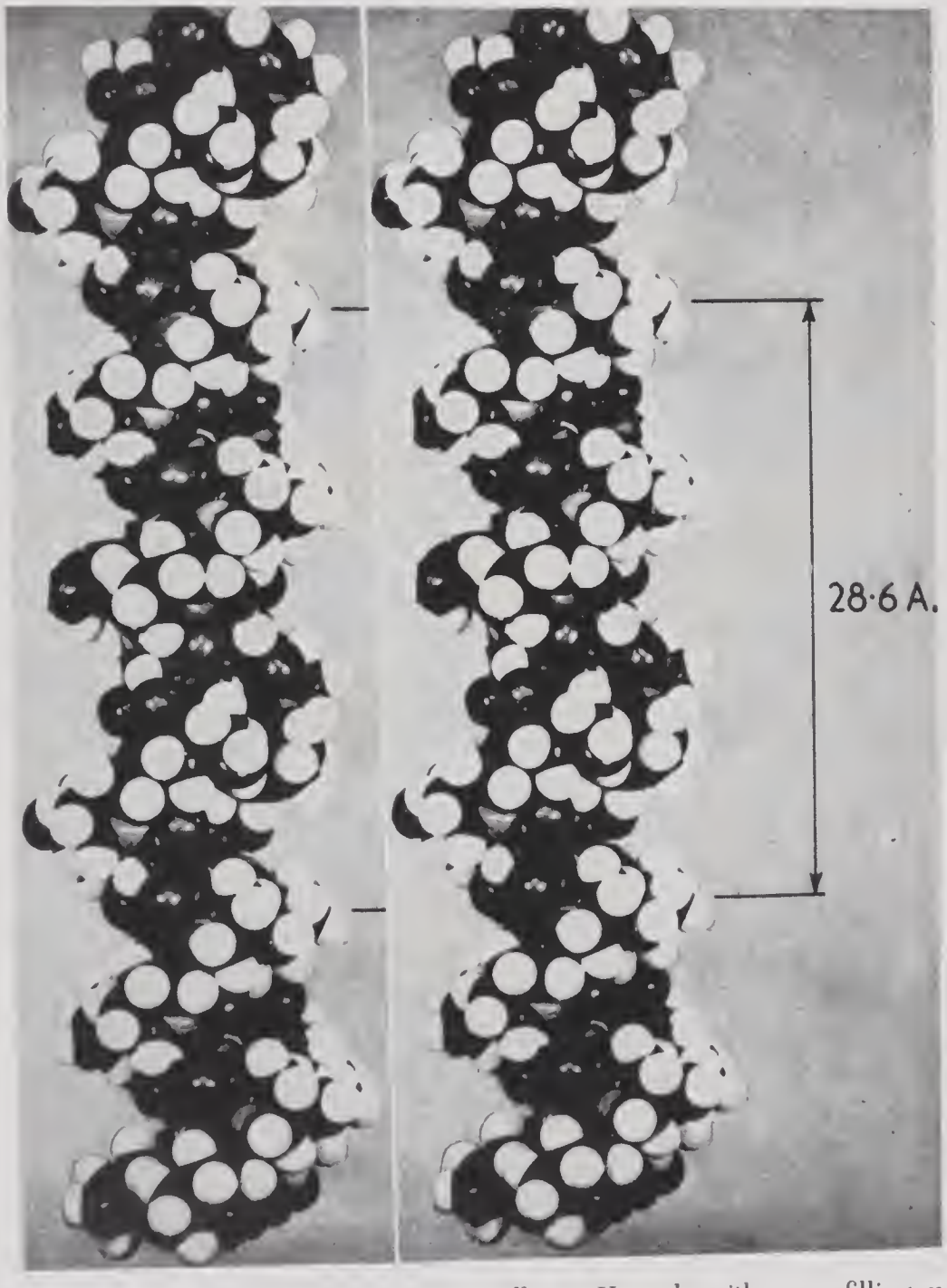


FIG. 21. Photograph of two models of collagen II made with space-filling models built with a repeat sequence Gly·Pro·Hypro. It may be seen how the ridge of one molecule tends to lie next to the groove of its neighbor. Courtesy of Rich and Crick (1961).

cule. The original studies by Schroeder *et al.* (1954) and Kroner *et al.* (1955) indicated that every third residue could be glycine and that the other two positions could be occupied either by proline and hydroxyproline or by any other amino acid. The recent elegant work of Grassmann and his associates on the isolation of peptides and the identification of the respective amino acid sequences is sufficient to establish the broad outlines of the primary structural pattern which predominates in the polypeptide chains of collagen; the magnitude of the task confronting workers in this field may be realized when one considers that there is something approaching the order of 3000 residues in each collagen molecule.

Grassmann *et al.* (1956, 1957, 1960) have used tryptic digestion and a combination of continuous curtain electrophoresis and column and paper chromatography to study the amino acid sequence in peptides derived from calf skin collagen. Of the homogeneous peptides so far analyzed, 90% have been shown to contain between 30 and 38% glycine, but three peptides consisting of 4 or 5 residues each have been found without glycine; the peptides constitute 55% of the amino acids present in collagen and range from tripeptides to one containing 131 residues. Grassmann *et al.* have suggested that 12 of the peptides isolated are probably triple-chain structures, since only 18 N-terminal groups have been found and both lysine and arginine are in the C-terminal positions. These observations suggest that there must be relatively stable interchain linkages in collagen. One of the isolated peptides had a chain length of 42–44 residues, of which almost exactly one-third was proline and hydroxyproline, one-third glycine, and the remaining third other amino acids. Many of the isolated peptides are either strongly polar, or nonpolar, which supports Bear's proposals for fiber structure (Bear, 1952).

Collagenase has also been used to liberate peptides ranging in weight from 500 to 600, but a few are larger; this enzyme has been shown to be highly specific, since in general it requires the sequence ·Pro·X·Gly·Pro· with cleavage occurring between X and Gly (e.g., Gallop *et al.*, 1957; Seifter *et al.*, 1959). The disadvantage of studies with collagenase is the small size of the peptides released, but both Schrohenloher *et al.* (1959) and Gallop and Seifter (1962) have demonstrated that two tripeptides, Gly·Pro·Hypro· and Gly·Pro·Ala· together account for 23% of the alanine, 14% of the glycine, 23% of the hydroxyproline, and 40% of the proline. Hannig and Nordwig (1963) have recently stressed the alternating character of imino-rich and imino-poor regions in the primary structure; purification methods have succeeded in showing that some of the nonpolar regions consist of peptides with

chain lengths of 6 to about 30 residues and that they may be broken down by collagenase digestion to release tripeptides of Gly·Pro·R·, which constitutes about one-third of the total amino acids.

e. Size and shape of the collagen molecule. The molecular weight of this molecule has been studied extensively, and at first it was believed to be of the order of several million (e.g., M'Ewan and Pratt, 1953). Subsequently, however, Boedtker and Doty (1956) found particles with a molecular weight of 360,000 in solutions of ichthyocol collagen; these particles behaved in solution as rigid rods, and the dimensions were 3100 A. in length and 13.5 A. in diameter. The diameter thus agreed well with that predicted by the wide-angle X-ray diffraction studies. Electron microscope investigations by C. E. Hall (1956) showed that these units had a mean length of 2200 A. and a diameter of about 15 A.; these results were in fair agreement with those of Boedtker and Doty. Furthermore, the existence of such particles, i.e., tropocollagen, had been postulated by Gross *et al.* (1954) from their studies of the precipitated products of collagen solutions.

On degradation of ichthyocol collagen under mild conditions, e.g., by gently heating at acid pH, a parent gelatin was obtained with a molecular weight average of 138,000 (Boedtker and Doty, 1956). These findings were thought to indicate that the monomer had broken into three fragments which showed a random coil configuration typical for isolated polypeptide chains (Cohen, 1955); thus the degradation of the molecular configuration must have occurred longitudinally. Gallop (1955) however, found a molecular weight of 70,000 for the parent gelatin and suggested that this particle represented the true collagen molecule.

f. The macromolecular architecture of the collagen fibril. Schmitt *et al.* (1942) and Wolpers (1943) were the first to demonstrate the presence of an axial periodicity in collagen fibrils; these observations were in direct agreement with those of Bear (1952) made by the use of low-angle X-ray diffraction. The characteristic period of most collagen fibrils consists of transverse striations asymmetrically grouped into bands and interbands so as to form a period of about 640 A. Metal shadowing of dry collagen fibrils shows that they have a corrugated outline (Fig. 15). While certain bands and interbands may be selectively stained with heavy metals, e.g., phosphotungstic acid and uranyl acid (Grassmann *et al.*, 1957, Hodge and Schmitt, 1960), density traces taken along the axis of untreated fibrils have demonstrated variations in density which represent different amounts of material; the regions of greater density correspond to more material which is localized in the band region (Burge and Randall, 1955). Investigations of developing tissue (e.g., Fitton

Jackson, 1956; Ross and Benditt, 1961) and of tissue cultures (e.g., Porter, 1952) have shown the presence of thin collagen fibrils which lack a distinctive 640 A. periodicity. In such fibrils the period usually measures 210 A. and appears to be uniform, as age increases the fibrils enlarge in diameter until a defined size for that tissue is reached and a 640 A. periodicity becomes apparent.

Many studies have been made of the way in which the collagen macromolecules form highly characteristic fibrils, and certain aspects of this problem will be briefly discussed. It has been known since the work of Zacchariadies (1900) that extracts of collagen could be obtained by means of dilute mineral acids, and in 1936 Wyckoff and Corey demonstrated that after precipitation of acid solutions of collagen by means of salt the typical wide-angle X-ray fiber diagram for collagen was obtained from the resultant precipitates. Later, Schmitt *et al.* (1942) showed by electron microscopy that such fibrils possessed the characteristic periodicity of collagen. The subsequent pioneer work of Schmitt, Gross, and Highberger (e.g., Highberger *et al.*, 1951; Gross *et al.*, 1954, 1955; and Schmitt *et al.*, 1955) demonstrated that the behavior of collagen in solution was very complex and that these soluble fractions could be precipitated by a variety of procedures to give different types of aggregates, as seen in the electron microscope. Other investigations included those of Orekhovich *et al.* (1948; Orekhovich, 1952), Fitton Jackson and Randall (1953), and Randall *et al.* (1952, 1953, 1955); the last authors stressed that a number of factors were involved in the precipitation of fibrils from solution and included the pH of the immediate local environment, the ionic strength, the concentration of protein in solution, the temperature, and the electrostatic charge profile of individual collagen molecules.

Three main types of aggregates were produced in these precipitation experiments; typical 640 A. banded fibrils (Figs. 22 and 23) obtained from acid solutions on dialysis against, for example, water of high pH or salt solution of precise molarity; fibrils with long spacings (F.L.S.) (Fig. 24) with a periodic structure of about 2400 A. which formed on addition of, say, glycoprotein to the solution; and third, fibrils with segment long-spacing (S.L.S.) (Fig. 25) formed most simply by the addition of a small anion, such as adenosinetriphosphoric acid to solutions of collagen. The demonstration of these three types of aggregates led Gross *et al.* (1954, 1955) to postulate the existence of a fundamental collagen particle, possibly equivalent to the collagen molecule, termed tropocollagen; they considered that this was the kinetic unit and consisted of a thin elongated particle having a length equal to the long

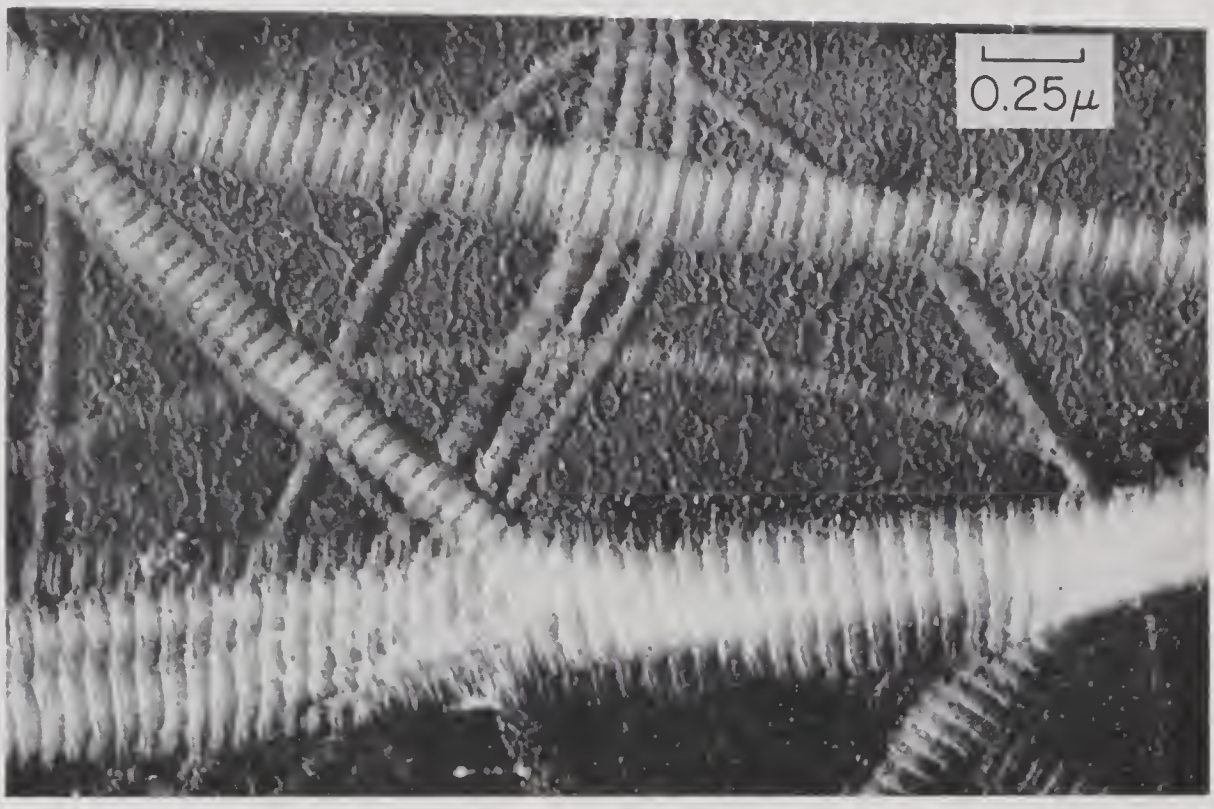


FIG. 22. Electron micrograph of collagen fibrils reprecipitated from an acetic acid solution of collagen by elevation to pH 7.0. Magnification: × 40,000.

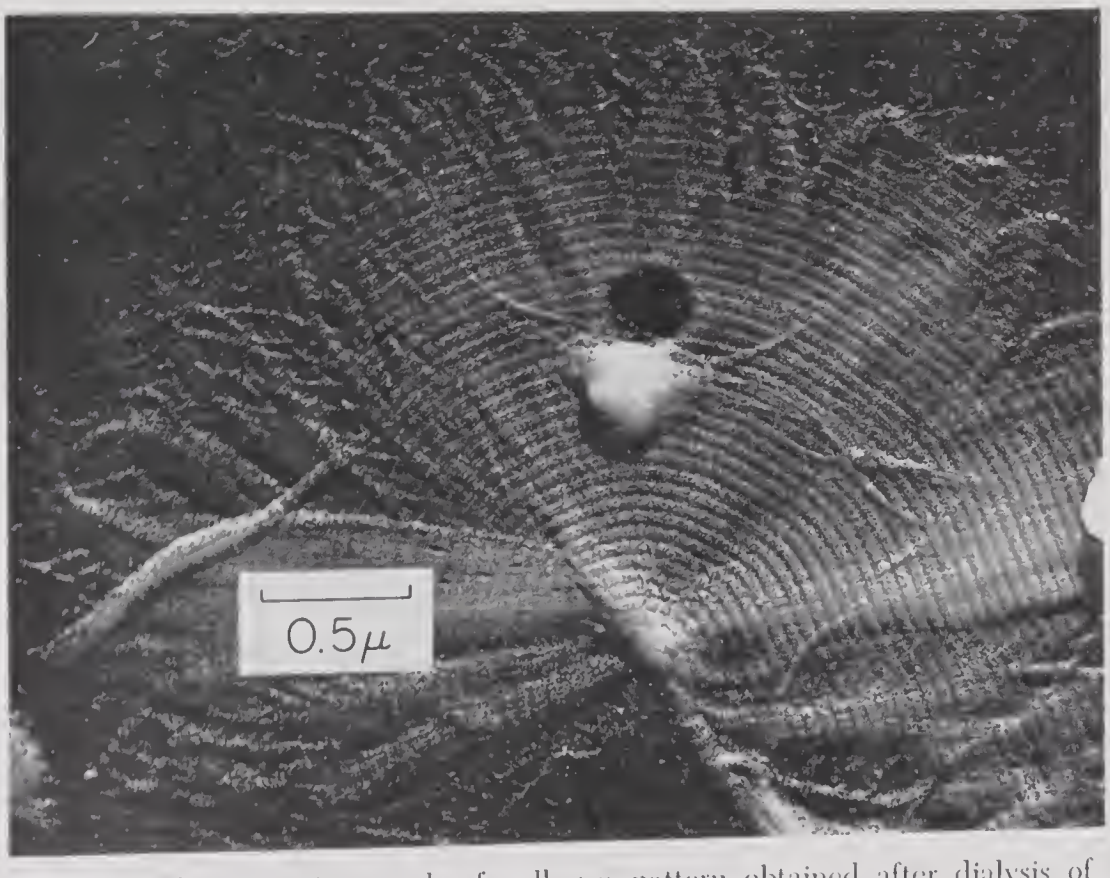


FIG. 23. Electron micrograph of collagen pattern obtained after dialysis of an acetic acid solution against sodium acetate. Magnification: × 25,000.

spacing and being capable of reversible conversion into native type collagen, F.L.S. or S.L.S., by combination in certain ways to form the particular aggregate. It was suggested that the native type of 640 A. period was the result of aggregates of tropocollagen units oriented all

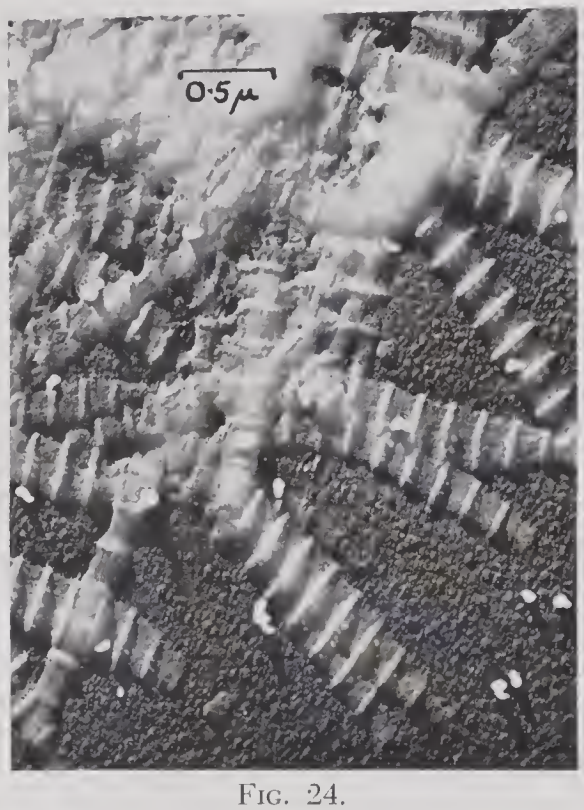


Fig. 24.

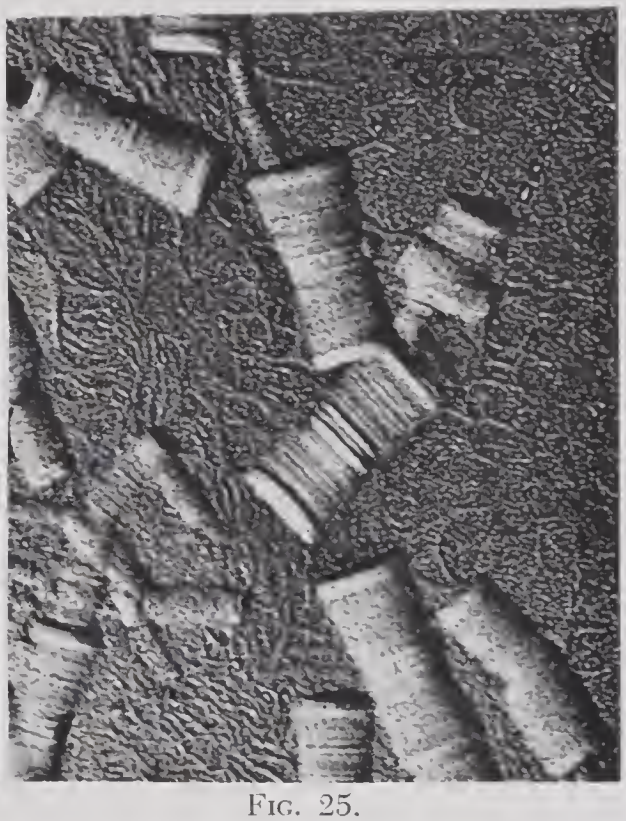


Fig. 25.

Fig. 24. Fibrous long-spacing organization of precipitate from an acetic acid solution with mucopolysaccharide. Magnification: × 48,000.

Fig. 25. Segmented long-spacing organization after precipitation of an acetic acid solution with adenosine triphosphoric acid. Magnification: × 48,000.

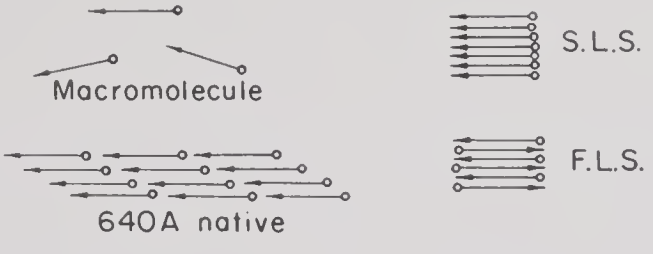


Fig. 26. Diagram illustrating the various arrangements of tropocollagen units to form the characteristic 640 A.; F.L.S. and S.L.S. type of fibril. After Gross *et al.* (1955).

in the same direction but staggered with respect to the macromolecular ends by specific lengths, thus giving a small repeat period; the F.L.S. fibrils were formed by an array of antiparallel units with their ends in register, whereas the S.L.S. forms were aggregates of units in parallel array with ends in register (Fig. 26). The prediction of the existence of the tropocollagen molecule was confirmed subsequently by the work

of Boedtker and Doty (1956) and C. E. Hall (1956), as mentioned previously.

Nishihara and Doty (1958) found that sonic irradiation of tropocollagen monomers in solution cleaved them into halves and quarters, which, however, retained the normal three-chain helical structure of the molecule; these findings imply that the molecular units of collagen must be about 77 A. in length, rather than the 3000 A.-long tropocollagen monomer. Studies of the fibrous forms of precipitates obtained from the sonic irradiated material led Hodge and Schmitt (1958) to postulate that the longitudinal aggregation of the macromolecules might "involve a specific type of coiling of terminal dangling chains about each other" and that the bonding system would be mainly H bonds. Indirect evidence suggested that the peptide appendages might contain the greater part of the tyrosine of the monomer (Hodge *et al.*, 1960). On the other hand, however, Grassmann *et al.* (1961) held that most of the tyrosine was an impurity—as suggested earlier by Eastoe and Leach (1958)—since extracts of collagen capable of forming segments (S.L.S.) appeared to contain only about 0.1%.

At the same time Hodge and Schmitt (1960) made a careful analysis of the charge profile of the tropocollagen macromolecular by high resolution electron microscopy and the addition of cationic and anionic heavy metal stains; in this way 80 striations were recorded, 40 being equivalent to bands and 40 to interbands. The theory of "quarter stagger" of tropocollagen monomers to reproduce the native type of periodicity was put to further test, first by using multiple exposures on a photographic plate with an appropriate displacement of the recording plate between successive exposures when a synthesized pattern was reproduced from the original segment (S.L.S.) which demonstrated a 700 A. periodicity. Second, heavy metal staining also indicated that there were four equivalent loci equally distributed within the tropocollagen monomer and that, if these four loci were quarter staggered with respect to each other in adjacent molecules, then the native type periodicity was reproduced. These findings thus supported the "quarter stagger" hypothesis and implied that there were four periods of equal length within the monomer.

Further work by Hodge (1963) in which the negative-staining technique and high resolution electron microscopy were used (Fig. 27), suggested, however, that the actual length of the tropocollagen macromolecule was 4.4 periods instead of 4.0 periods, and, instead of a "quarter stagger" or overlap of 1 period in adjacent tropocollagen macromolecules, as previously proposed, the overlap was only 0.4 of a period followed by a gap of 0.6 of a period. Thus the longitudinal aggregations of tropo-

collagen macromolecules would cover 5 periods per monomeric unit. Hodge observed alternating light and dark areas superimposed on the main band pattern, these zones coinciding exactly with the overlap of the ends and the sites of the holes; the overlap region extended from the A_3 to the c_2 band, in the general native pattern (Fig. 28) and hence the holes must lie between the c_2 and a_2 bands. If this pattern of alignment is valid, it implies that the three-dimensional structure of the fibril must be built up from sheets of tropocollagen monomers, in which the 0.4 period overlap occurs, and that the overlap pattern holds only for fibrils immediately above and below in adjacent layers. These observations suggest that the lateral bonding of adjacent molecules is of paramount importance; since the size of the hole must be at least 350 A. chemical bonding for end-to-end aggregation of adjacent molecules must be ruled out. This suggests that a secondary substance within this region may be present, a point that will be discussed later in the chapter.

The implication of Hodge's observation is that all three chains stretch the full length of the tropocollagen macromolecule; this requirement is of interest in view of the point stressed by Grassmann *et al.* (1963) that all chain ends must be at the same level as a prerequisite for the specific renaturation of the molecule from individual chains. On the other hand, Rubin *et al.* (1963) have presented further evidence for the existence of a peptide chain that facilitates end-to-end aggregation of the molecules beyond the triple helical body of the tropocollagen monomer by showing that intramolecular bonding occurred in a pepsin-labile end region of the monomers. Nishihara *et al.* (1963) have suggested that pepsin attacks at about 250 A. from the end of the tropocollagen particle, and it is interesting that the amino acid composition of the peptide material released by pepsin is strikingly different from that of collagen (Rubin *et al.*, 1963).

The 10% pepsin labile material may be equivalent to the overlap regions proposed by Hodge (1963), and the present author wonders whether this region is composed of noncollagenous protein such as may be found in the ground substance (cf. p. 407). Tristram and Steven (1962) have reported the presence of thirteen amino acids reacting as N-terminal residues which are derived from a protein-poor nitrogen fraction forming about 2% of the total protein nitrogen, and which were physically associated with soluble collagen. If gaps of the order of 350 A. occur between the ends of adjacent collagen macromolecules, it would seem most likely that some other substance may be incorporated at this point, at least during the process of fibrogenesis *in vivo*.

g. The stability of the collagen fibril. The relative structural im-

portance of cross-links between individual polypeptide chains of single molecules and those between adjacent molecules is not known, but there is evidence that the rigidity of the molecule depends upon the intactness of all three polypeptide chains. The importance of H-bonding and

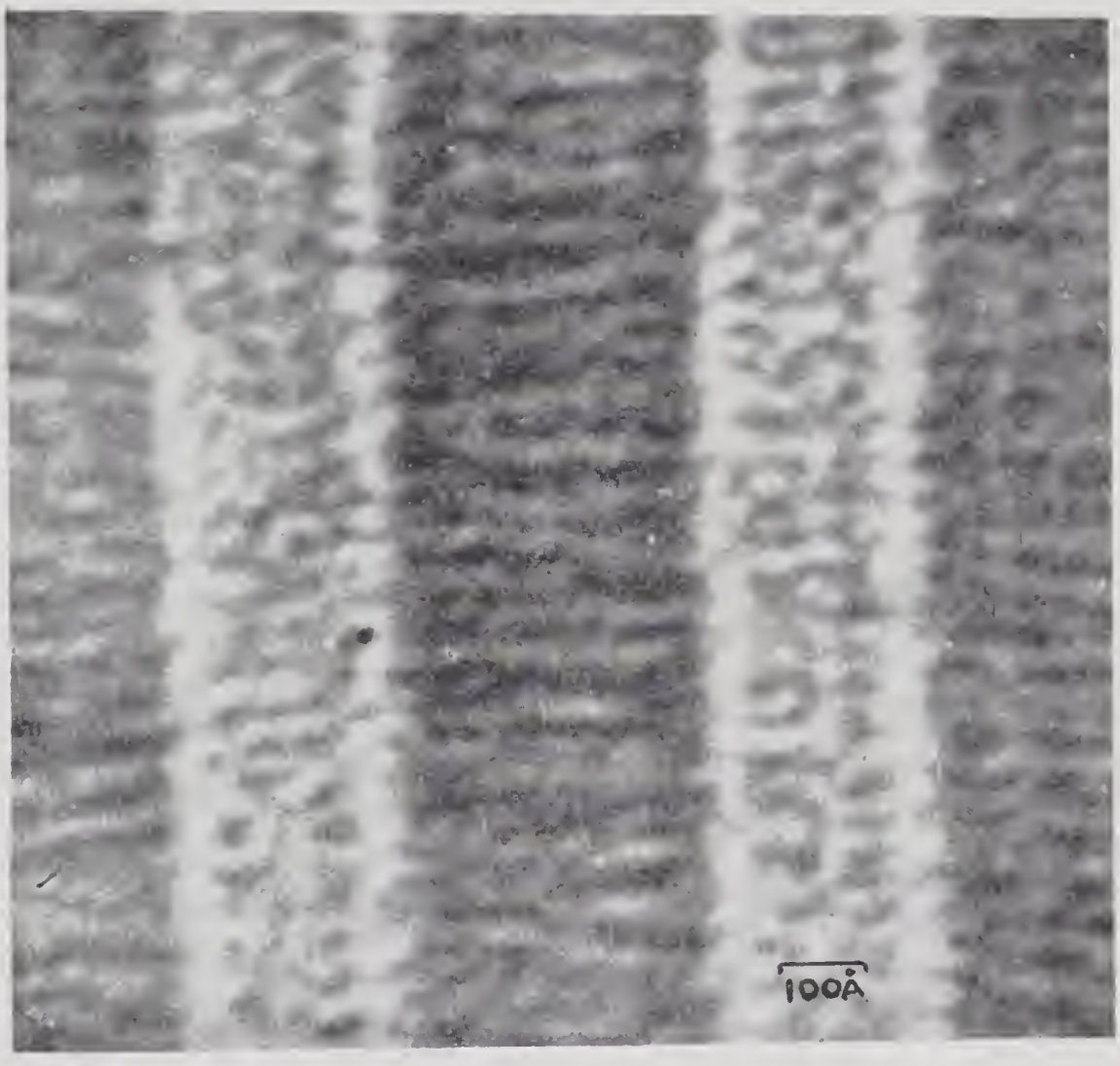


FIG. 27. Negative-stained image of a short piece of a collagen fibril in which just over one period of 640 A. is visible. Note the arrangement of the individual protofibrils clearly seen in the main interband region. Magnification: × 880,000. Courtesy of Tromans *et al.* (1963).

peptide and amino peptide bonds in the maintenance of the molecular configuration has been discussed earlier; at present it is not known whether the hydrogen bonding in which hydroxyproline is involved is intra- or intermolecular; the latter would increase structural stability. The possibility that other bonds are also concerned in the maintenance of structure has been investigated. It has been suggested that about one-third of ε-amino groups of lysine may be masked in some form

of peptide chain cross-link (e.g., Bowes and Kenten, 1948), and studies by Hörmann (1962) indicate that they are intramolecular.

Grassmann *et al.* (1954) suggested that ester links might exist between carbohydrate and the hydroxyl group of hydroxyamino acids, and a number of other studies have been made with respect to this. Gallop *et al.* (1959, 1960) examined the selective cleavage of ester bonds by hydroxylamine in parent gelatin; they concluded that esterlike bonds existed and that the specific disruption released fundamental subunits of the collagen molecule of a molecular weight about 25,000. From these data Gallop *et al.* concluded that 12 such units would be required to form a tropocollagen monomer; evidence suggested that intramolecular ester

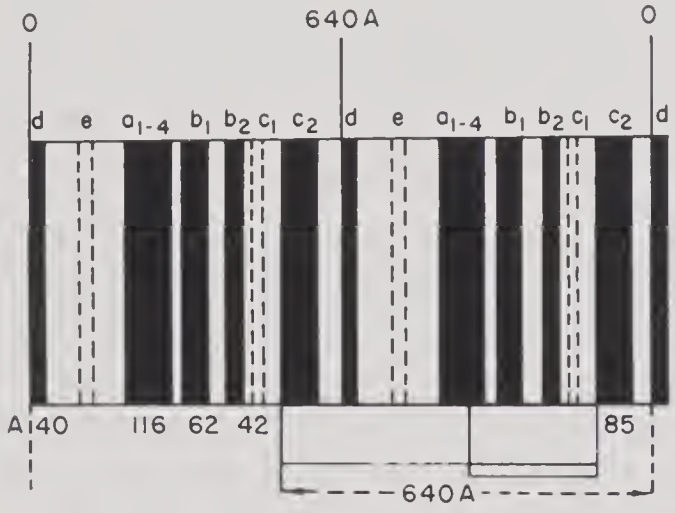


FIG. 28. Diagram of the main bands and interbands within two adjacent periods of a collagen fibril showing the approximate position of the "gap" between the c_2 and a_2 bands.

links occurred in pairs not further apart than 7.7 residues. Gallop *et al.* also carefully examined the possibility that carbohydrate was concerned in the bonding, and they finally decided that it was not involved (Seifter, 1963).

Studies of the properties of the various soluble collagens and the insoluble fraction have shown that the degree of cross-linking varies with the type of the tissue and the species of animal from which it is derived; furthermore, it has been shown to increase progressively with age not only at the molecular level *in vitro* (e.g., Gross, 1959), but also in tissue from aged specimens. This latter observation suggests that interfibril bonding may also be of some importance. Finally it must be recalled that D. S. Jackson (1953) suggested that the small amount of sugar which appears to be an integral part of the collagen fibril may act as a stabilizing agent.

h. The kinetics of chain aggregation. Two structural problems associated with the formation of collagen fibrils *in vivo* are, first, the manner whereby the macromolecules are aligned into fibrils, and, secondly, whether the three polypeptide chains are generated as single chains A, B, and C and then are spontaneously aggregated to form a macromolecular unit ABC, presumably through some form of H-bonding, or whether generation of all three chains to form a cohesive unit ABC occurs simultaneously. It can be seen from the previous discussion that the arrangement of the macromolecular units within the collagen fibril is becoming clearer, and recent work by Wood (1962) has suggested that a nucleation period was followed by a growth period that resulted in the formation of fibrils. Recent work on renaturation of collagen solutions has shed some light on the second problem of the phenomenon of aggregation of polypeptides into macromolecules.

Orekhovich and Shpikiter (1955) studied the sedimentation characteristics of citrate-soluble collagen in 3 *M* urea after preliminary heating at 30°C. for 10 minutes; they concluded that the collagen monomer was a complex of two components, α and β, which in the native structure were originally linked by relatively weak bonds; further work led to the evaluation of the molecular weight of the α component as 80,000 and of the β component as 130,000 (Orekhovich and Shpikiter, 1958). Nishihara and Doty (1958) and Chun and Doty (1958) noted that after more extensive treatment of collagen solutions of various skins, the β component could be split into two α units; there was a corresponding marked reduction in viscosity and change in optical rotation of these solutions; this finding led to the proposal that the β component was in fact composed of two polypeptide chains of similar size. This hypothesis thus implied that the triple chain configuration of collagen was made up of three α chains, two being bonded together to form the β unit, and that the third α chain was twined around this unit. Amino acid analyses by Piez *et al.* (1960), however, showed that the compositions of the β and α components were different, a finding which suggested that if in fact the β unit was made up of two α chains, then the composition of the α chains must vary.

In studies of the renaturation of gelatin solutions by means of cooling, Veis and Cohen (1960) and Rice (1960) found that about 10% of the parent gelatin reverted to rodlike structures and that at least some of this fraction was capable of forming segments (S.L.S.) as seen by electron microscopy, implying that intact tropocollagen molecules were present. Algelt *et al.* (1961) investigated this phenomenon in greater detail and showed that this fraction, named γ, was capable of rapid

renaturation and formed into segments (S.L.S.); it was suggested that the γ fraction was composed of three polypeptide chains which remained linked by covalent bonds even in the denatured state so that they behaved as a single kinetic unit.

Grassmann *et al.* (1963) have studied the renaturation process in detail and found that individual chains can be induced to re-form into the correct monomeric configuration by a spontaneous process at low temperature. In order to reconstitute the tropocollagen molecule as opposed to nonspecific aggregates, however, it is necessary to obtain the correct alignment so that the ends of the combined triple chains are in

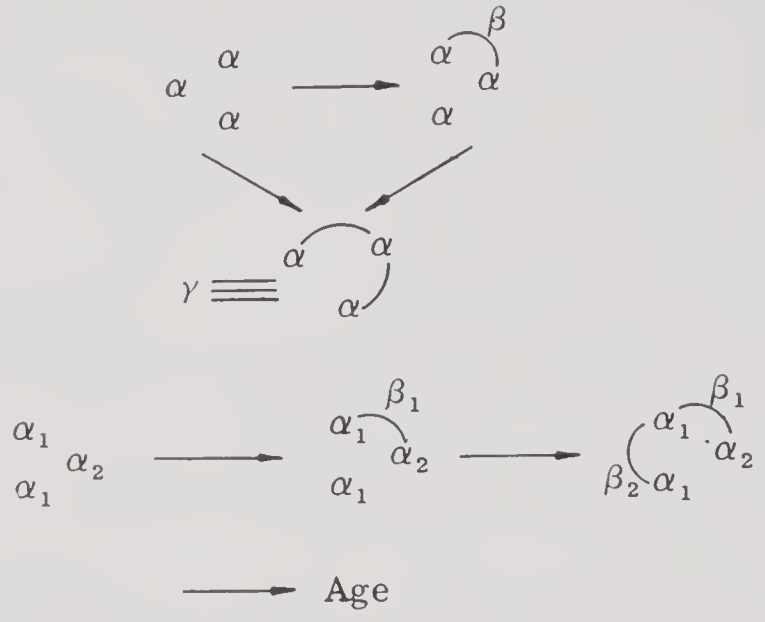


FIG. 29. Diagram illustrating the relationship between the different subunits of the collagen molecule. Based on Grassmann *et al.* (1961) and Piez *et al.* (1963).

the same place. In completely denatured solutions, these conditions can only be obtained by alternating the temperature from 4°C. to 22°C.; such denaturation and renaturation gradually produces a transition from nonspecific aggregation to a specific tropocollagen type of aggregation. The components after renaturation are mainly of the α and γ type, and Grassmann *et al.* suggested that the native molecules can be formed from either three α subunits or from one β and one α subunit. They also recorded the presence of a component with double intramolecular bonds. These suggestions of Grassmann *et al.* have been set out in Fig. 29.

In a further study Piez *et al.* (1961) compared the properties of two denatured solutions (40°C.) of rat skin collagen, one having been prepared initially by extraction with 1 *M* sodium chloride and the other by 0.5 *M* acetic acid. The results showed that salt-extracted collagen sedimented as one large boundary while acid-extracted collagen gave two

boundaries characteristic for α and β components. Subsequent chromatography of these solutions on carboxymethylcellulose at 40°C. demonstrated that the acid-extracted fraction was composed of four fractions designated α_1, α_2, β_1, and β_2 whereas the salt-extracted fraction was composed essentially of α_1 and α_2 in a ratio of 2:1 (Fig. 30). Further separation of the β_1 and β_2 components from the acid-extracted fraction showed that β_1 consisted of α_1 and α_2 subunits in equal amounts whereas β_2 was composed of α_1-type subunits. Amino acid analysis of the different

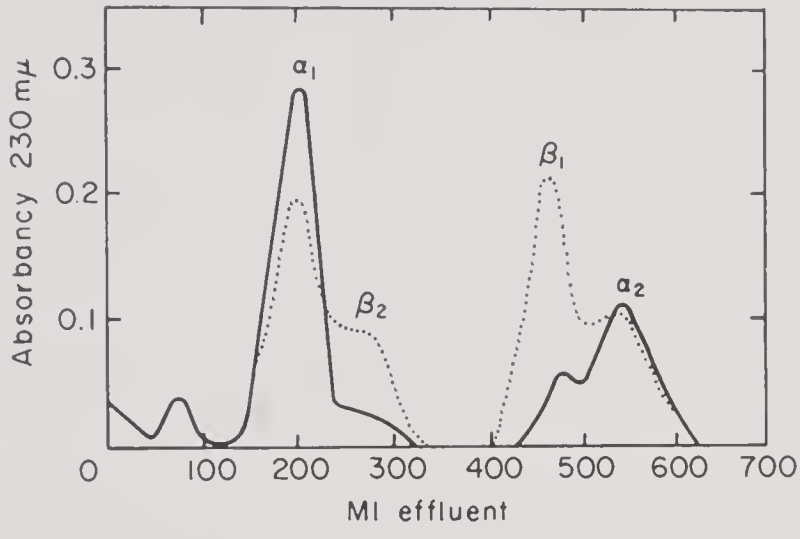


Fig. 30. Diagram showing the separation of the collagen macromolecule into four fractions after treatment on cellulose acetate column. Courtesy of Piez *et al.* (1961).

subunits showed that the composition was different (Table VI). Piez and colleagues suggested that the newly formed collagen, i.e., neutral salt soluble, consisted of three subunits of approximately equal size, two of which were α_1 and one of which was α_2, and that the subunits represented single chains, each of which might extend the whole length of the molecule. The acid-extracted material represented more mature molecules, in which two units were covalently cross-linked to form β components (Fig. 29), thus agreeing with the original suggestion made by Orekhovitch and Shpikiter (1958).

These studies on the specific renaturation of three polypeptide chains to form the highly characteristic monomer imply that the electric charge and its distribution on the individual chains may be primary factors in reaggregation; they have demonstrated also the heterogeneity of the collagen macromolecule especially at the subunit level. It is becoming clearer that many complicated steps are concerned in (1) chain aggregation and (2) macromolecular aggregation to form fibrils; the motivating forces involved, however, are not yet understood. Whether the

hypothesis derived from the studies of the mode of aggregation of the partieles discussed above, can be applied directly to processes of fibrogenesis as it oecurs in the tissue is open to experiment. The evidence obtained from these studies is invaluable in providing an insight into the type of phenomena that may be concerned, but tissue fibrogenesis may well require a more complex series of activities during the aggre-

TABLE VI
AMINO ACID COMPOSITION OF THE SUBUNITS OF RAT SKIN COLLAGEN[a,b]

Amino acid	Original	Subunits			
		β_2	α_1	α_2	β_1
3-Hydroxyproline	9	1.5	1.0	0.0	0.7
4-Hydroxyproline	92	97	96	86	88
Aspartic	46	45	46	44	44
Threonine	19.6	19.8	19.9	19.8	20.1
Serine	43	41	42	43	42
Glutamic acid	71	72	74	66	70
Proline	121	128	129	113	120
Glycine	331	333	330	336	338
Alanine	106	111	112	102	107
Valine	24	19.2	19.6	32	25.6
Methionine	7.8	6.6	8.0	6.1	7.0
Isoleucine	10.8	6.5	6.4	16.1	11.6
Leucine	23.8	18.7	18.1	32.4	25.7
Tyrosine	2.4	1.8	2.1	2.4	2.2
Phenylalanine	11.3	12.1	11.6	10.1	11.0
Hydroxylysine	5.7	3.9	4.3	8.0	6.2
Lysine	28.1	30.1	30.4	22.4	25.8
Histidine	4.9	2.0	1.9	8.5	5.3
Arginine	51	50	49	50	50
Amide N	(41)	(41)	(42)	(42)	(42)

[a] Data taken from Piez *et al.* (1963).
[b] Values: residues per 1000 residues.

gation process. These may include the necessity of an activating agent such as an enzyme as well as interaction with other materials contributed by the ground substance.

i. Biosynthetic pathways in the formation of collagen. i. Hydroxylation mechanism. Studies of the biosynthesis of the collagen macromolecule are facilitated by the presence of two hallmarks, namely hydroxyproline and hydroxylysine. The initial experiments of Stetten and Schoenheimer (1941) showed that when N^{15}-labeled proline was fed to rats it was readily incorporated into the body protein; a significant part of the label appeared in the hydroxyproline fraction, the specific activity of which was a little lower than that of proline. Further experiments by

Stetten (1949) demonstrated that N^{15}-labeled hydroxyproline was not incorporated into the newly formed body protein. These results led Stetten (1949) to suggest that "the hydroxyproline of proteins is not derived to an appreciable extent from dietary hydroxyproline, but rather from the oxidation of proline which is already bound, presumably in peptide linkage." The oxidation of both proline and lysine therefore might well constitute an important step in the sequence of reactions involved in the formation of the collagen macromolecule.

Van Slyke and his associates found that lysine was the chief source of hydroxylysine in the collagen of young rats fed with C^{14}-lysine; the relative rates of labeling of the two imino acids suggested that the hydroxylation step occurred simultaneously, or nearly so, with incorporation into the collagen (Sinex and Van Slyke, 1955). Further investigations demonstrated that feeding of labeled hydroxylysine to young rats failed to lead to the incorporation of significant amounts into skin collagen (Sinex *et al.*, 1959). Van Slyke and Sinex (1958) noted that the ratio of specific activities of hydroxylysine to lysine was 0.68, 0.71, and 1.10 in collagen from rats killed 1 hour, 6 hours, and 2 weeks after injection of C^{14}-lysine; such results might be interpreted as evidence for a gradual hydroxylation of lysine after incorporation into the collagen macromolecule. In a further investigation Popenoe and Van Slyke (1962) took great care, after suitable injections of labeled isotope into young rats, to obtain collagen of high purity; they demonstrated that the activities of lysine and hydroxylysine were in fact equal to each other at all times.

An investigation of the rapid synthesis of collagen in cultures of osteoblasts led Smith and Fitton Jackson (1957) to confirm Stetten's observation that hydroxyproline was derived directly from proline; they noted, also, that C^{14}-(L)-glutamic acid was incorporated by the cultures, but no significant amount was converted to hydroxyproline. When neutral salt-soluble collagen was extracted from slices of granuloma induced by carrageenin in guinea pigs, following incubation in the presence of C^{14}-(L)-proline the ratio of the specific activity of hydroxyproline to proline increased from approximately 0.6 to 1.6 during a 4-hour period (Green and Lowther, 1959). The authors pointed out that a ratio greater than 1 was difficult to reconcile with the suggestion of bound proline undergoing oxidation. It is of interest that Robertson *et al.* (1959) proposed that such a ratio might indicate that the imino acids entered the collagen polypeptides as separate entities and hence that there was both an "active hydroxyproline" and an active proline pool, the former derived from the latter. On the other hand, Hausmann and Neuman (1961) concluded that the ratio of the specific activity of hydroxyproline

to proline was unity in fractions of soluble collagen extracted from guinea pigs into which C^{14}-proline had been injected. If proline were incorporated into peptide linkage before hydroxylation occurred, it is probable that a proline-rich peptide would be formed first; attempts to isolate such a unit, however, have been inconclusive (Robertson *et al.*, 1959; Gould *et al.*, 1960).

Further work on the biosynthesis of collagen has been concerned with the examination of various cellular fractions with a view to identifying the site of hydroxylation. Lowther *et al.* (1961) reported the presence of highly labeled, peptide-bound hydroxyproline in a microsomal fraction obtained from guinea pig granuloma induced by carrageenin; the specific activity of the hydroxyproline in both the supernatant and mitochondrial fractions was much less. In studies on the intracellular localization of collagen synthesis in the intact chick embryo, Prockop *et al.* (1962) again found that protein-bound hydroxyproline with the highest specific activity was present in the microsomal fraction derived from homogenates of the chick embryos. This work was extended to investigate the cell-free biosynthesis of collagen; Peterkofsky and Udenfriend (1963) employed double labeling of proline with tritium and C^{14} and time studies with puromycin and ribonuclease. Their observations led them to conclude that the substrate of hydroxylation was a microsomal RNA-bound polypeptide of considerable size.

On the other hand Gould and Manner (1963) have proposed from studies of the behavior of labeled proline and lysine in cell-free sRNA fractions obtained from 9–11-day chick embryos that both proline and lysine are converted to active sRNA complexes of hydroxyproline and hydroxylysine, respectively. Studies by Meister *et al.* (1963) also are in accord with this view; in their work they demonstrated that proline was converted to collagen proline and collagen hydroxyproline by two separate and distinct pathways, since a striking fall in the specific activity occurred when collagen hydroxyproline was isolated from ascorbic acid-deficient granulomas, as compared with controls. The significance of ascorbic acid in the formation of collagen has been reviewed by Gould (1960).

Thus it is still an open question whether hydroxyproline and hydroxylysine are incorporated into collagen protein by mechanisms involved in many forms of protein synthesis, or whether the hydroxylation step entails a polypeptide intermediate of considerable size.

ii. Particle size and distribution of bound hydroxyproline within cells. Precise data are not available of either the size or the physical character of "collagen" units which are synthesized in the cells, although it is assumed frequently that a particle of at least the size of tropocollagen is

contained within the cell; such a macromolecular unit would be the product of the aggregation of smaller subunits. Studies of the distribution of bound hydroxyproline in different cellular fractions, however, have provided some relevant information. Analyses of four cellular fractions obtained after homogenization and differential centrifugation, in 0.88 *M* sucrose of collagen-producing tissue cultures showed that much of the bound hydroxyproline was in two "large granule" fractions which were undoubtedly of cellular origin (Fitton Jackson, 1958, 1960). The first

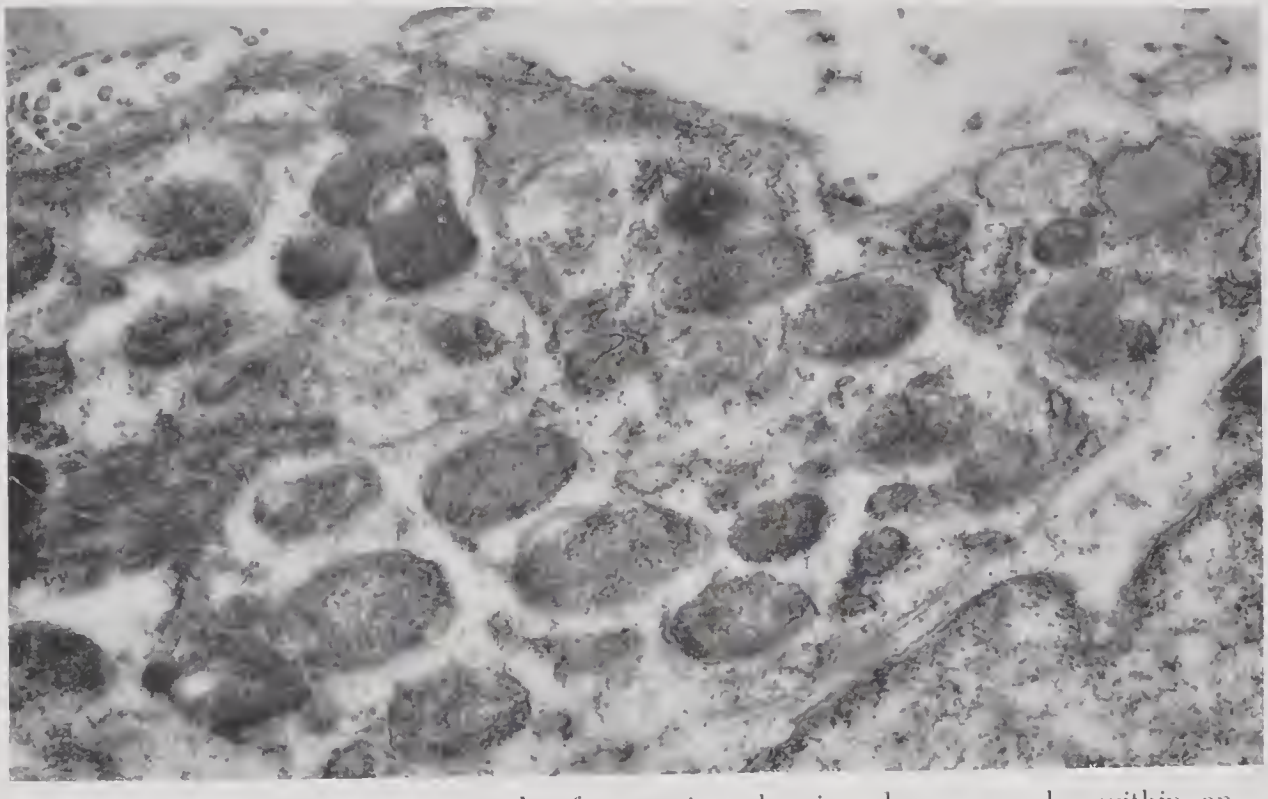


FIG. 31. Electron micrograph of a section showing dense granules within an osteoblast that constitute the greater part of the second "large granule" fraction, obtained from cultures of frontal bone after 48-hour growth. Magnification: × 35,000.

"large granule" fraction was essentially equivalent to a mitochondrial fraction, whereas the second "large granule" fraction was of lower molecular weight with a calculated particle size of 3000 A., a spherical shape being assumed. Dense granules of varying internal structure (Fig. 31) were observed by electron microscopy in sections of this material, but characteristic collagen fibrils were not seen. With increased age of the cultures, most of the bound hydroxyproline became localized in the residue fraction that represented the extracellular phase but included also some cytoplasmic material as well as nuclei; characteristic collagen fibrils, however, were not observed in this fraction during the first 24 hours of growth and were not present in whole cultures at this age (Fitton

Jackson and Smith, 1957). A small amount of bound hydroxyproline was also in cellular fractions of low molecular weight similar to a "clean" microsomal fraction. It is of interest that in the 12-hour cultures more than 50% of the total bound hydroxyproline was located within the cell.

Prockop *et al.* (1961) found that the bulk of the hydroxyproline (6.43 μmoles) was present in a 15,000 *g* cellular fraction obtained from 9-day-old chick embryos; the microsomal (0.10 μmole) and soluble (0.15 μmole) fractions contained considerably less; it was established that the hydroxyproline was contained in these fractions in a nondializable peptide form. In studies of tissue slices of carrageenin granuloma, Lowther *et al.* (1961) noted that a high proportion of a microsomal fraction (0.25 *M* sucrose at 105,000 *g*) was extracted by 0.14 *M* NaCl and suggested that it was comparable to newly synthesized collagen. Analysis of this material by Eastoe (1961) showed that the amino acid pattern was essentially that of collagen; electron microscopy (Lowther *et al.*, 1961) indicated that the fraction consisted largely of particle-studded vesicles from disrupted endoplasmic reticulum, but filamentous units were not observed.

These studies uphold that a collagen-like material is present within the cells and may accumulate in fractions of large-granule size, but morphological evidence is lacking about the state of molecular aggregation of this material.

j. The turnover of collagen. Under normal physiological conditions the collagen of most connective tissues is relatively inert, as demonstrated, for instance, by the rate of incorporation of C^{14}-glycine into liver, bone, skin, and tendon of rats (Neuberger and Slack, 1953). In some tissues, however, collagen may be reabsorbed swiftly, as occurs during the involution of the uterus. The rate of turnover of collagen in the tissues that have been examined to date may be correlated with the solubility properties of various collagenous fractions. As first shown by Harkness *et al.* (1954) an alkali-soluble fraction was evidently a younger form of collagen than either the acid-soluble or insoluble fractions. An investigation by D. S. Jackson (1957a,b) demonstrated that during the development of subcutaneous granulomata induced by carrageenin in guinea pigs, a neutral salt-soluble fraction was the first to appear after an initial lag period of 3 days; after the addition of C^{14}-glycine, the specific activity of the isotope was found to be much higher in this fraction during the next 24 hours than in either that of the acid-soluble or insoluble fractions; 24 hours later, however, the activity of the three fractions became almost identical.

Subsequent work has indicated that the neutral salt-soluble collagen

may be divided into classes based on salt concentration used in the extraction procedure. Gross (1959) proposed that cold physiological salt solution (0.14 *M* NaCl) extracted collagen that was unaggregated or only loosely aggregated whereas hypertonic solutions (e.g., 0.45 *M* NaCl) also extracted collagen recently cross-linked in fibrillar form. Dilute organic acids were capable of extracting the neutral salt-soluble collagen as well as that more heavily bonded. Earlier, Gross (1958a) had noted that the total amount of neutral salt-soluble collagen in the skin of young guinea pigs formed about 10% of the total collagen in the dermis and represented 1–2-days growth of new collagen.

The work of D. S. Jackson and Bentley (1960) provided data for a similar scheme; the skin and carrageenin granulomata of guinea pigs were extracted progessively with neutral salt solutions of increasing

TABLE VII
THE EXTRACTION OF COLLAGEN FROM GUINEA PIG SKIN BY VARIOUS EXTRACTING MEDIA[a]

M NaCl (pH 7.4)	Collagen extracted (as mg hydroxyproline)		Specific activity (c.p.m./μmole glycine)	
	Single fraction	Accumulative total	8 Hr	36 Hr
0.14	10.2	10.2	1970	625
0.28	17.5	27.7	1060	528
0.45	15.1	42.8	710	692
1.0	9.2	52.0	370	615
2.0	5.1	57.1	300	512
Citrate (pH 3.6)	13.2	70.3	62	88

[a] Data from D. S. Jackson and Bentley (1960).

ionic strength. Analyses of the content of hydroxyproline and the specific activity of previously injected C^{14}-glycine in different fractions demonstrated that after 8 hours the specific activity decreased with increased ionic strength of the extractant; after 36 hours, however, a leveling out process took place (Table VII). These data were held to indicate that variations in the extractability of neutral salt solutions were dependent upon the time that had elapsed since macromolecular formation; differences noted in the granulomata results suggested that a certain degree of degradation occurred in the tissue. It is necessary, perhaps, to take into account that the apparent heterogeneity of neutral salt-soluble collagen may represent different stages of collagen chain formation prior to the aggregation of completed units of tropocollagen as well as stages subsequent to macromolecular formation. This suggestion is supported by the biosynthetic studies, mentioned previously, in which it was found that

the specific activities of certain amino acids varied in different particulate fractions of cells.

The differences in metabolic behavior and physical characteristics of the various fractions of collagen have led to the hypothesis that a continuous spectrum of macromolecular aggregation, increasingly strongly cross-linked is present in connective tissue. Even in young animals the proportion of metabolically active, neutral salt-soluble collagen is small; much of the collagen is insoluble and hence metabolically inert. It is interesting, therefore, that a recent study by Popenoe and Van Slyke (1962) showed that the percentage of injected C^{14}-lysine retained in skin collagen of weanling rats indicated so slow a turnover of the insoluble fraction during a 15-week period that it was impossible to measure it accurately. As mentioned previously, a continuous increase in the number of cross-links within the collagen fibrils is believed to be the most characteristic feature of the aging process in connective tissue (e.g., Gross, 1961). The metabolic inertness and insolubility of the bulk of the collagen supports this hypothesis.

2. *Reticular Fibers*

The available evidence supports the concept that there is no structural difference between reticular and collagen fibers. Robb-Smith (1958) has postulated that there are two types of reticular fibers, those of the basement membrane reticulin which are extremely stable, and immature argyrophilic fibers, found in embryonic and regenerating areas of connective tissues, and which appear to be incorporated within collagen fiber bundles during growth. It is believed that the material surrounding the reticular fibers accounts for the characteristic silver and periodic acid–Schiff reactions of these fibers.

3. *Elastin*

The possibility that the same cells synthesize the material for the formation of both collagen and elastic fibers has long been considered by histologists, since no distinctive elastoblast has been described. Elastic fibers bear little resemblance to collagen fibers. For example, their histological staining properties are very different; the elastic fibers are highly extensible, whereas collagen fibers are not; elastic fibers are resistant to water heated at 120°C., whereas collagen fibers are readily soluble; elastic fibers cannot be converted into gelatin, and the amino acid analyses of the two substances show substantial differences. Recently Partridge has written an excellent review of the properties of elastin (1962).

The fact that collagen is often found in close association with elastin led to the suggestion that collagen could be converted into elastin (Hall,

1959), but the bulk of the evidence is in favor of a separate identity. As will be seen later, there is no basic reason why the same cell should not be capable of synthesizing these different products.

III. The Cytology of Connective Tissue Cells

A. Cell Types and Cellular Differentiation

Most collagenous connective tissues have a common origin in the embryonic mesenchymal cell, which in the course of differentiation forms the connective tissue proper, cartilage, and bone; the cells that are actively concerned in the development and maintenance of these tissues are fibroblasts and fibrocytes, chondroblasts and chondrocytes, and osteoblasts and osteocytes, respectively. Cytodifferentiation in connective tissues consists of an orderly progression of predictable changes which lead to the production and maintenance of the extracellular materials; as mentioned in the Introduction, a fundamental assumption must be made that these connective tissue cells are each capable of synthesizing all the various products required for a given tissue. It is not known, however, whether such activities are carried out simultaneously or in alternating waves, but it is probable that the immediate functional state of the cell is dependent upon environmental requirements. Such statements imply that connective tissue cells are pluripotential in the sense that they possess the required genetic information for a number of diverse specialized activities.

Other types of cells in connective tissue include histiocytes of macrophages, which may act as scavengers by the ingestion of extraneous particles in the tissue; mast cells, usually grouped around the blood vessels lying within the connective tissue, with their cytoplasmic complement of granules believed to contain heparin and histamine (e.g., Sylven, 1957; Asboe-Hansen, 1957); leucocytes, lymphocytes, and plasma cells; in addition pigment and fat cells are scattered throughout the loose and dense connective tissues.

That the process of cellular differentiation consists of a sorting out of heterogeneous cytoplasmic particles into recognizable structural features which are then capable of a specific function, is evident from various studies of the morphogenesis of connective tissues; for instance, the cytoplasm of the mesenchymal cells that are predestined to form membrane bone in the avian embryo, i.e., preosteoblasts, is less highly organized than that of the subsequent differentiated cells, i.e., osteoblasts, that are laying down bone matrix (Fitton Jackson and Randall, 1956a). Little is known, however, about the ways in which the different primary chemical units in the cell are formed into complex cytoplasmic entities, each with a particular function.

The various pathways of differentiation open to connective tissue cells have been a subject for much debate; detailed histological studies of the differentiation of bone cells in normal development led Pritchard (1952, 1956) to conclude that they belonged to the same race of specialized mesenchymal cells and that the fibrous periosteum formed isolated centers, within which the cells retained osteogenic and chondrogenic capacities. Undifferentiated mesenchymal cells may be present in adult connective tissues and act as replacements for differentiated cells that have degenerated, but their presence is a controversial issue, since they are not easy to identify. It has been suggested frequently that active connective tissue cells, e.g., fibroblasts, in adult tissue are derived from, e.g., histiocytes or other perivascular cells, but it is extremely difficult to design suitable experiments to settle this issue.

Most of the theories of cellular differentiation in connective tissue have rested, of necessity, on the interpretation of events based on many meticulous observations of histological preparations (cf. Maximow and Bloom, 1957). The demonstration by Davidson *et al.* (1963) that the synthesis of acid mucopolysaccharide by a cell line of connective tissue origin falls immediately when DNA-dependent RNA synthesis is blocked by actinomycin D, provides a valuable approach to the study of the genetic control of specialized cellular activities. The use of tritiated thymidine provides a method of tracing the fate of any cells that were synthesizing DNA at the time of application of the isotope, but certain reservations must be made in the interpretation of results in view of, for example, the possibility of subsequent breakdown of DNA and reutilization of these products.

Tonna (1960) studied with tritiated thymidine the proliferative rate of various skeletal tissues in mice; the autoradiographs showed that the preosteoblasts of the periosteum were frequently labeled and that they gave rise to cells required for the expansion of the epiphyseal disk. Young (1962) traced the transformation of proliferating cells into specialized bone cells in endochondral osteogenesis in 6-day-old rats by autoradiography in studies with thymidine-H^3. He concluded that cell division was restricted to preosteoblasts (termed osteoprogenitor cells by Young) and that after division cells may either remain in a preosteoblastic pool or continue "modulation" into osteoblasts or osteoclasts. Young found that the turnover of osteoblasts varied in different regions of bone formation but was of the order of 2 days; subsequently the cells either continued to differentiate into osteocytes, or returned to the preosteoblast pool, as suggested by Heller *et al.* (1950). The results of Owen (1963) were essentially in agreement with those of Young; she noted that the osteoblasts remained nearly 3 days on the surface of new

rabbit bone before they differentiated into osteocytes with enfoldment into the new bone. The grain counts in the various cells, however, suggested that many preosteoblasts did not divide during the period of observation (Owen and MacPherson, 1963).

The origin of the multinucleated osteoclasts, concerned in processes of bone destruction, has been open to question since the original observations of von Kölliker (1873); osteocytes, chondrocytes, osteoblasts, and preosteoblasts have all been implicated, as well as endothelial leucocytes (cf. Hancox, 1949, 1956). The work of Young (1962) supports the view that osteoclasts are derived by fusion of preosteoblasts; he noted a progressive appearance of labeled nuclei in osteoclasts from 9 hours after injection with tritiated thymidine; the results suggested also that there may be a turnover of nucleated components in the osteoclasts. Studies, with tritiated thymidine, of regenerating limbs of the newt by Fischman and Hay (1962) have led to the conclusion that osteoclasts arise by fusion of mononuclear leucocytes, probably monocytes, between 9 and 20 days after injection of the isotope; it was not clear, however, whether preosteoblasts might not contribute also to the formation of osteoclasts since appropriate regenerates were not examined later than 5 days after labeling. On the other hand, istotope studies by Messier and Leblond (1960) implicated the osteocyte as the main source of osteoclasts; since the complement of enzymes of both osteocytes and osteoblasts is markedly different to that in osteoclasts (Burstone, 1960), the fusion of these cells to form osteoclasts seems unlikely unless a striking change in cellular economy first occurs. On balance, the available evidence supports the contention that both preosteoblasts and monocytes take part in the formation of osteoclasts.

B. Morphological Characteristics and Properties

The above discussion supports the contention that cells actively engaged in the formation and maintenance of connective and skeletal tissue are but modulations of the same stem cell; many studies of cytodifferentiation are compatible with the supposition that alterations in the cytochemical properties and fine structure of various cellular organelles are associated with changes in their functional state, and some of the evidence will now be examined.

1. The Shape of the Cells

Cellular shape largely depends upon the nature of the extracellular substance. In general fibroblasts are elongated and somewhat irregularly star shaped with an ovoid nucleus surrounded by a thin sheath of cyto-

plasm; several cytoplasmic processes stretch out and many branch and establish contact with those of neighboring cells, thus forming a cellular net, through the meshes of which collagen fibers interwind. Osteoblasts range from 15 to 80 μ in size, the amount of cytoplasm increasing with differentiation; they are mainly oval in shape, one side resting on the surface of the new bone that is being laid down by the cell. The rounded nucleus often lies near the free cell surface, and thin cytoplasmic processes of extreme length penetrate the bone and anastomose with those of neighboring cells. Chondroblasts, which vary in size depending on the type of cartilage, are oval in form and have a relatively large nucleus; there is very little cytoplasm in prechondroblasts, but the amount increases markedly with age and thin cytoplasmic processes penetrate the surrounding cartilage matrix. In contrast to fibroblasts and osteoblasts, the surface contour of chondroblasts shows many large indentations. Fibrocytes, osteocytes, and chondrocytes are all somewhat similar in appearance to the blastic cells from which they are derived, and from which they differ mainly in their smaller size and flattened form.

2. *The Cell Surface*

Electron microscopy of sectioned connective tissues has demonstrated that the definitive surface of the cell is represented by a triple-layered structure seen as two dense lines each about 25 A. wide separated by a less dense region of about 25 A. Because of many small invaginations the surface may not be clearly defined in all parts of the cell and appears sometimes to be replaced by regions of dense material which may also be observed just within, and continuous with, the intact cell surface. In the early stages of differentiation, the cells of some connective tissues appear to form a syncytium, e.g., in tendon (Fitton Jackson, 1956) and in cartilage (Godman and Porter, 1960); it would seem probable, however, that the cells are independent of each other, but that the delicate surface is not easy to distinguish at this stage.

The structure of the cell surface in connective tissues is of particular relevance since the respective surfaces must be capable of permitting the passage of substances of different molecular weights both into and out of the cell; such movement may be conditioned by the requirements of the cellular processes being carried out since a reciprocal balance must be maintained between the intake of various substances and intracellular synthesis, and the expulsion of material to form the extracellular phase. Unfortunately few data concerning the mechanisms involved are available; observations of pincytotic activity and the dynamic nature of the cell surface in cultures of connective tissue cells are well documented,

but the precise localization of, for example, enzymatic activity at the cell surface, as observed by Mölbert *et al.* (1960) in the surface layer of epithelial cells of rat kidney tubules awaits further experimentation.

3. The Mitochondria

Active connective tissue cells are characterized by many mitochondria, whereas the number is reduced in, for example, fibrocytes. Mitochondria in living cells demonstrate marked plasticity of shape and, in osteoblasts, are often localized at the periphery of the Golgi zone (Fell, 1932) but are usually randomly disposed in preosteoblasts. Electron microscopy shows that the internal cristae of the mitochondrion tend to be short and project transversely and irregularly into the internal matrix, which is denser in earlier stages of differentiation. Mitochondria increase considerably in size with differentiation in chondroblasts (Godman and Porter, 1960) and in fibroblasts of embryonic mouse skin (Kajikawa, 1961); the internal cristae frequently appear deranged and such changes in fine structure may reflect differences in enzymatic activity (e.g., see Lehninger, 1959; Sickevitz, 1959). Of importance to bone metabolism is the observation that mitochondria take up calcium rapidly *in vitro* (e.g., de Luca, 1962), and on the basis of recent studies utilizing electron microscopy Green (1963) has reported within the mitochondria the presence of crystals that appear to be a hydroxyapatite.

4. Cytoplasmic Granules

Cytoplasmic granules, about 0.3–1 μ in width are a striking feature of many connective tissue cells; neutral red-staining granules were first recorded by Dubreuil (1910) in osteocytes and were found in tissue cultures of osteoblasts by Fell (1932). Stearns (1940) reported that in repair tissue small granules of unknown composition were extruded from fibroblasts into the intercellular areas, where they appeared to take part in the formation of small collagen fibers. Heller-Steinberg (1951) has since demonstrated Schiff-positive granules in frozen-dried preparations of metaphyseal bone cells of the rat and has associated their presence with the deposition of new bone, while earlier Gersh and Catchpole (1949) had postulated the existence in fibroblasts of very small intracellular granules of a glycoprotein nature. A systematic investigation of the properties of connective tissue cells from embryonic tendon, bone, and cartilage, and of similar tissues grown in culture confirmed the presence of cytoplasmic granules (Fig. 32), but the number per cell in embryonic tissue were less than in the corresponding cultures (Fitton Jackson, 1955; Fitton Jackson and Smith, 1955; Fitton Jackson and Randall, 1956a). The

findings indicated that the granules contained protein and polysaccharide components, and it was suggested that they might have fibrogenic potency.

Cinemicrophotographic studies, at high magnification, of such cells in life showed that the granules were not extruded from the cells, but that they moved freely within the cytoplasm as individual discrete bodies. Electron micrographs demonstrated some dense particulate granules in

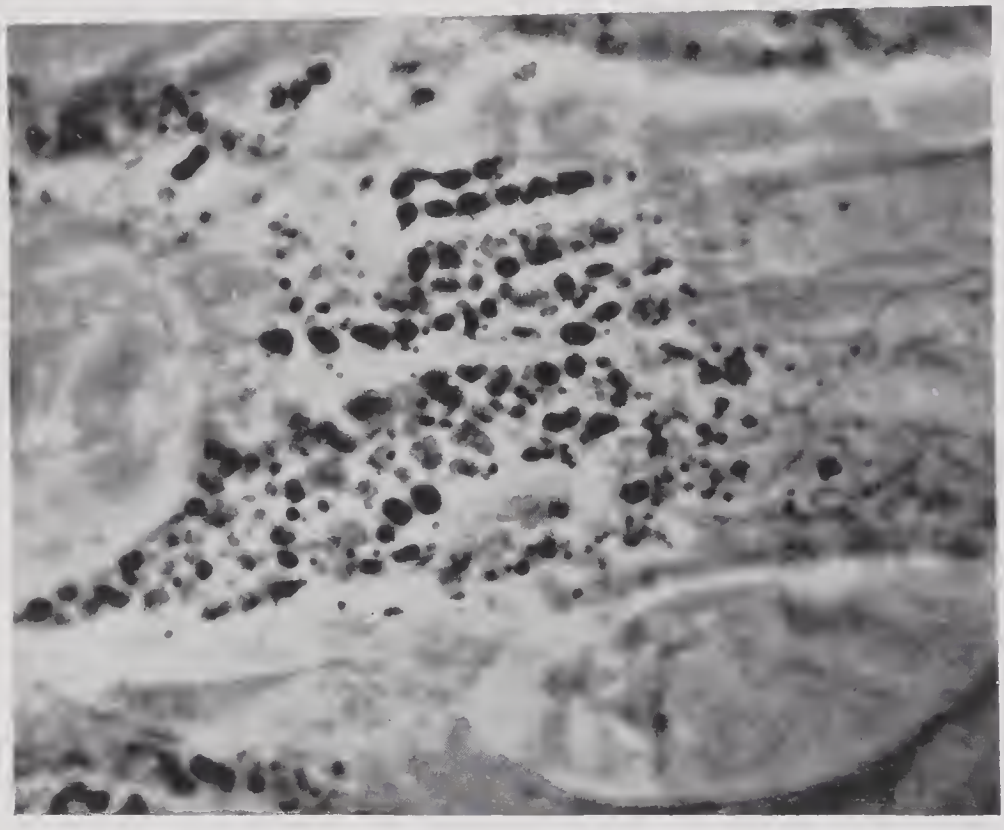


FIG. 32. Phase-contrast photomicrograph of part of an osteoblast growing from a living culture of frontal bone. Many cytoplasmic granules are clustered near the nucleus and filamentous mitochondria extend into the peripheral zone of the cytoplasm. (Fitton Jackson and Randall, 1956a). Magnification: × 1900.

the cultured cells (Fig. 31), but similar granules were not identified in the cells from embryonic tissue. Since the granules were discrete bodies and were seen to move about freely in the cells both in culture and in squashes of the embryonic tissues, it is clear that they cannot correspond to the dilated cisternae of the endoplasmic reticulum as suggested by Sheldon (1959) and D. A. Cameron (1961), and as discussed by Chapman (1962).

It is interesting that Robbins *et al.* (1955) also recorded the presence of cytoplasmic granules in dermal fibroblasts in culture and thought that they might represent an intracellular precursor of collagen fibers, since

the number of granules and amount of collagen formed diminished when the cultures were grown in the presence of anticollagen serum.

Some of the granules mentioned above showed variations in density when viewed in the living state by phase contrast, an observation which suggested that different granules may have diverse properties and functions. It is of interest, therefore, that recent work on "fibroblasts" in tissue cultures derived from the brains of puppies (Ogawa *et al.*, 1961) and from the ovaries of Chinese hamsters (Munro *et al.*, 1963) have been shown to contain acid phosphatase in cytoplasmic granules (Fig. 33).

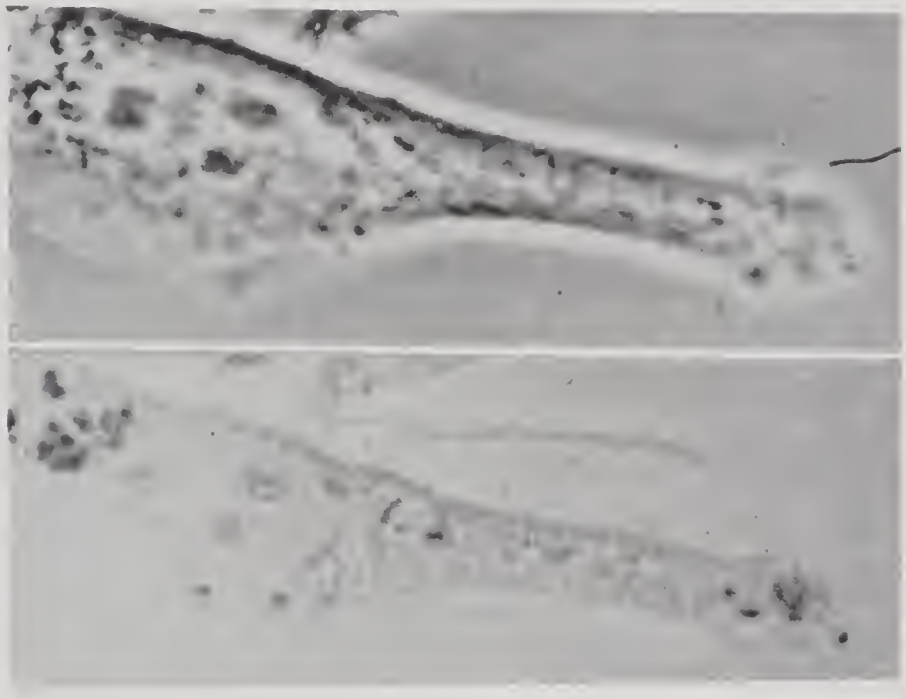


FIG. 33. Phase-contrast photomicrograph of an interphase cell grown in a culture of the ovaries of Chinese hamsters. Top: living cell; bottom: the same cell stained for acid phosphatase (Gomori) and counterstained with eosin. Courtesy of Munro *et al.* (1963). Magnification: × 1500.

Mulnard (1961) also recorded the presence of granules that stained metachromatically and contained acid phosphatase in embryonic chicken cells in culture. It has been suggested that these granules may be related to lysosomes (de Duve *et al.*, 1955). In these experiments, however, there was no evidence that constituents of connective tissue were being laid down, whereas in the work of both Fitton Jackson (e.g., 1955) and of Robbins *et al.* (1955) extracellular materials were being actively formed by the cells and there was a correlation between the number of granules present and the production of these substances. It would seem, therefore, that many granules observed in active connective tissue cells are distinct from lysosomes, but it is possible that some may be potentially lysosomal in character.

5. *The Golgi Zone*

Most connective tissue cells contain a distinctive region in the cytoplasm, adjacent to the nucleus, and earlier referred to as the juxta-nuclear vacuole, but now recognized as synonymous with the Golgi zone of the cell. Histological preparations indicated that this region consisted of discrete, but closely packed granules in osteoblasts (Fell, 1932). Observations of thin sections of various connective tissue cells by electron microscopy, show that the Golgi zone (Fig. 34) is composed of a mass of small, rounded vesicles with varying internal density and a complex of elongated sacs, sometimes closely stacked together, each sac being about 1 μ in length and bounded by a smooth membrane. With cytodifferentiation, large vacuolated vesicles become a prominent feature of this region, and studies by phase-contrast cinemicrophotography of osteoblast grown in culture (Rose, 1961) suggest that droplets of various sizes and low optical density arise in the Golgi zone and are not derived by pinocytotic activity. Electron microscopy shows that in chondroblasts these large vesicles appear to be bounded by a single, dense membrane (Godman and Porter, 1960), whereas in osteoblasts the bounding membrane is frequently double, each membrane being less than 40 A. thick and separated from each other by a clear area up to 100 A. wide (Fitton Jackson and Randall, 1956a).

For many years the Golgi zone of the cell has been linked with secretory activity; since an enlargement of vesicular bodies occurred during the process of cytodifferentiation (Fig. 36) and appeared to parallel the development of an extracellular phase in cartilage of embryonic rats, Godman and Porter (1960) suggested that the constituents of the ground substance were amassed within these vesicles which were subsequently extruded from the cell. Autoradiographic studies of the development of various connective tissues have indicated that certain relevant isotopes after their administration become localized rapidly within the cells and then move into the intercellular areas (see Fig. 47b–d, p. 493). Dziewiatkowski and colleagues have noted such a sequence of events with the use of S^{35}-sulfate (Dziewiatkowski and Campo, 1962). Leblond and associates have traced the fate of a number of labeled amino acids, and Carneiro and Leblond (1959) have recorded the presence of C^{14}-glycine within a Golgi-like zone of odontoblasts followed by its release into the dentinal matrix. The secretory nature of the Golgi zone has been stressed by Revel and Hay (1963) in a combined autoradiographic and electron microscope study of cartilage regeneration (referred to further in the section on chondrogenesis). There is no strong evidence, however, that the Golgi zone is concerned in the synthesis of any specialized

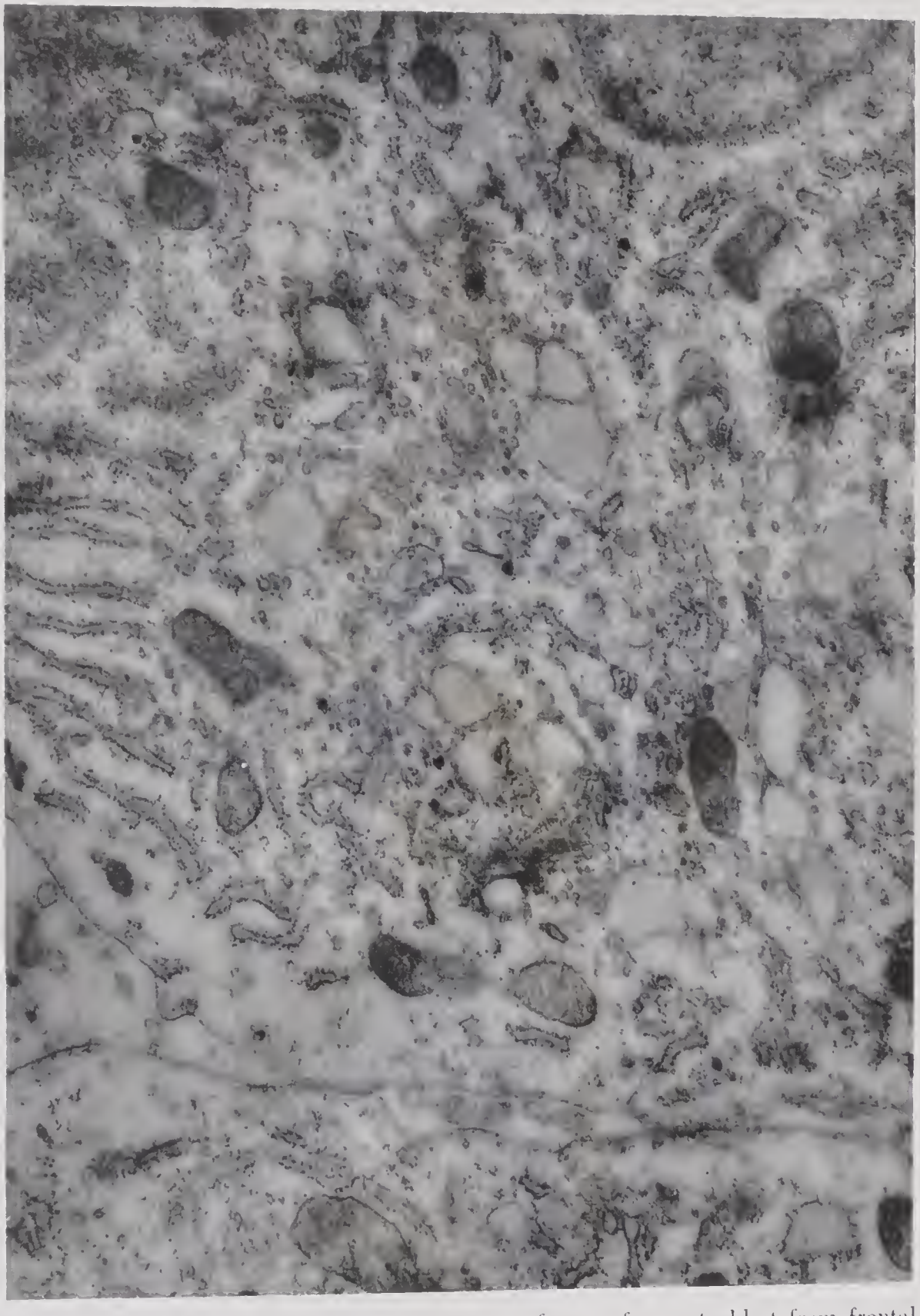


FIG. 34. Electron micrograph of a section of part of an osteoblast from frontal bone of a 13-day fowl embryo. The central region of the micrograph represents the Golgi zone of the cell; mitochondria, ergastoplasm, part of the cell surface, and intercellular spaces are well defined. Note organized ribosomal particles at top (left) of micrograph. Magnification: × 32,000.

product. Furthermore, the function of the Golgi zone may not be primarily secretory, since this region of the cytoplasm is well defined in nonsecretory cells. Oberling (1959) has postulated that the zone may function as an osmoregulator.

6. The Ergastoplasm

During the development of connective tissue much of the cytoplasm of the respective cells becomes intensely basophilic; the fine structure

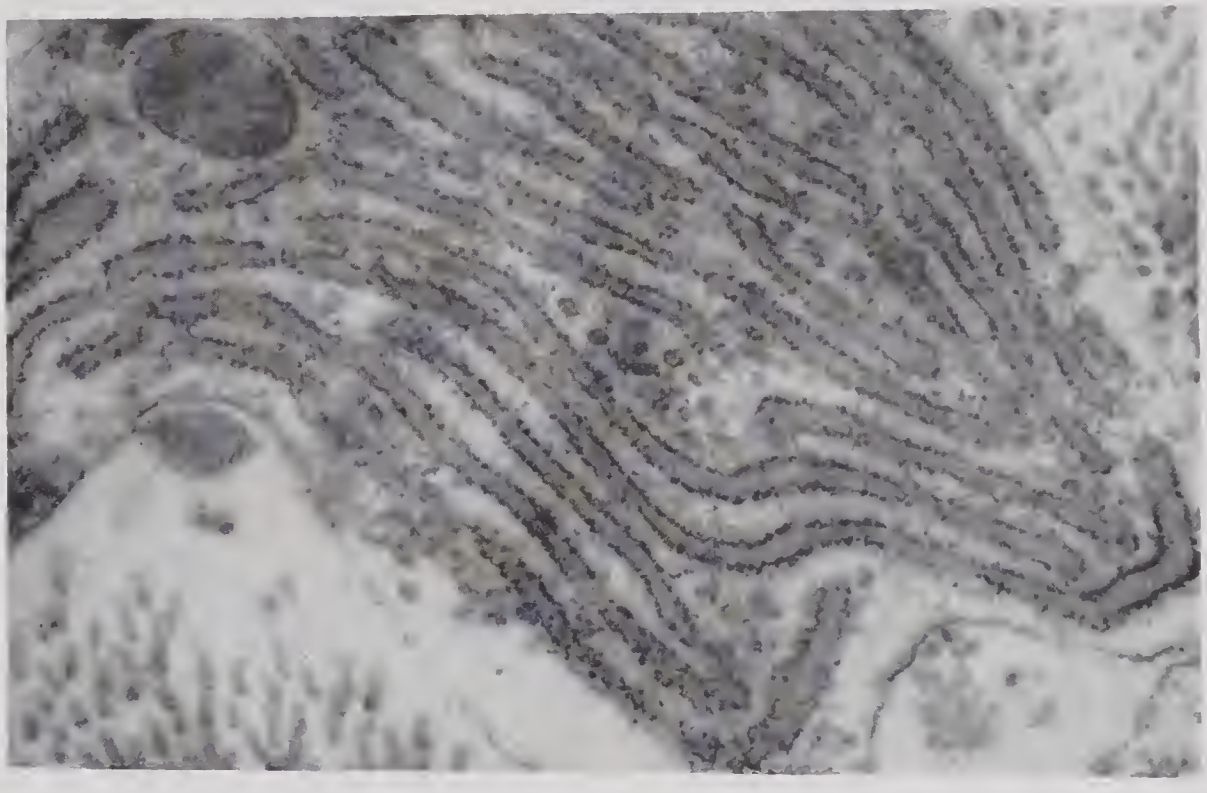


FIG. 35. The ergastoplasm in part of a fibroblast seen in a section of tendon from 14-day fowl embryo. Note the extreme density of the internal regions of the ergastoplasmic structures. Magnification: × 45,000.

of this region, referred to as the ergastoplasm, has been described in a number of studies (Fitton Jackson, 1956; Scott and Pease, 1956; Knese and Knoop, 1961a, b; Godman and Porter, 1960; Hay, 1958; Revel and Hay, 1963). In young tissue, the cells are weakly basophilic, and electron microscopy shows that the cytoplasm contains many dense particles, 100–200 A. in width, some of which are arranged in parallel lines, a few elongated profiles consisting of paired, smooth membranes closely opposed to each other, and a random distribution of small vesicles of varying internal density, each bounded by a membrane. An increase in basophilia of the cytoplasm during cytodifferentiation is paralleled by the appearance of many ergastoplasmic profiles (Fig. 35). This system con-

sists essentially of paired membranes, and numerous particles (ribosomes) associated with their external surface; the membranes are sometimes only 600 A. apart, but this distance may increase to form large cisternae, a feature observed especially in chondroblasts (Godman and Porter, 1960). In embryonic tissues the substance between the membranes is markedly dense compared with that of the surrounding cytoplasmic sap (Fitton Jackson, 1956); the ergastoplasmic profiles branch but, although they are seen close to the cell surface, a distinct interconnection between the ergastoplasmic membranes and the unit membrane of the cell surface has not been described. The general organization of the ergastoplasm is essentially similar in different types of connective tissue cells, but variations in the thickness of the membranes, in the size of the attached particles, and in the degree of density of the enclosed substance are apparent. Furthermore the amount of ergastoplasmic profiles may be different in adjacent cells lying in areas of rapid extracellular development, suggesting that not all cells are in the same metabolic state.

The available evidence is now overwhelming in favor of ergastoplasmic structures being concerned in certain stages of protein synthesis; membranous components provide enormous surface areas in the mature cell and could permit a precise spatial organization of associated ribosomes, and possibly polysomes, in relation to this surface. Moreover, it is probable that enzymes and/or messenger RNA required in the synthetic stages involving ribosomal particles, are arranged in precise assemblies on these surfaces and thus facilitate synthesis in ordered sequences. At early stages of differentiation, however, when synthesis of the specialized products is being actively pursued, ribosomes and groups of ribosomes are free in the cytoplasm, so that a provision of a surface is not a prerequisite for protein synthesis (Fitton Jackson and Randall, 1958).

The problem of the way in which the substances synthesized in association with the ergastoplasmic structures are moved about in the cell is of fundamental importance. Two general concepts have been proposed: that the internal channels of the ergastoplasm are used as passageways and may interconnect with the Golgi zone, thus permitting the transfer of material between the two organelles (Porter, 1957; Palade, 1958), and that the membranes may flow in the cytoplasm and thus transport associated substances (Bennett, 1956). The presence of numerous small vesicles in the cytoplasm suggests that they might provide a means of transport, and each might well contain a specific substance. Possible ways of controlling their movement are unknown.

The development of the ergastoplasm during maturation of the cell upholds the hypothesis that cytodifferentiation is accompanied by marked

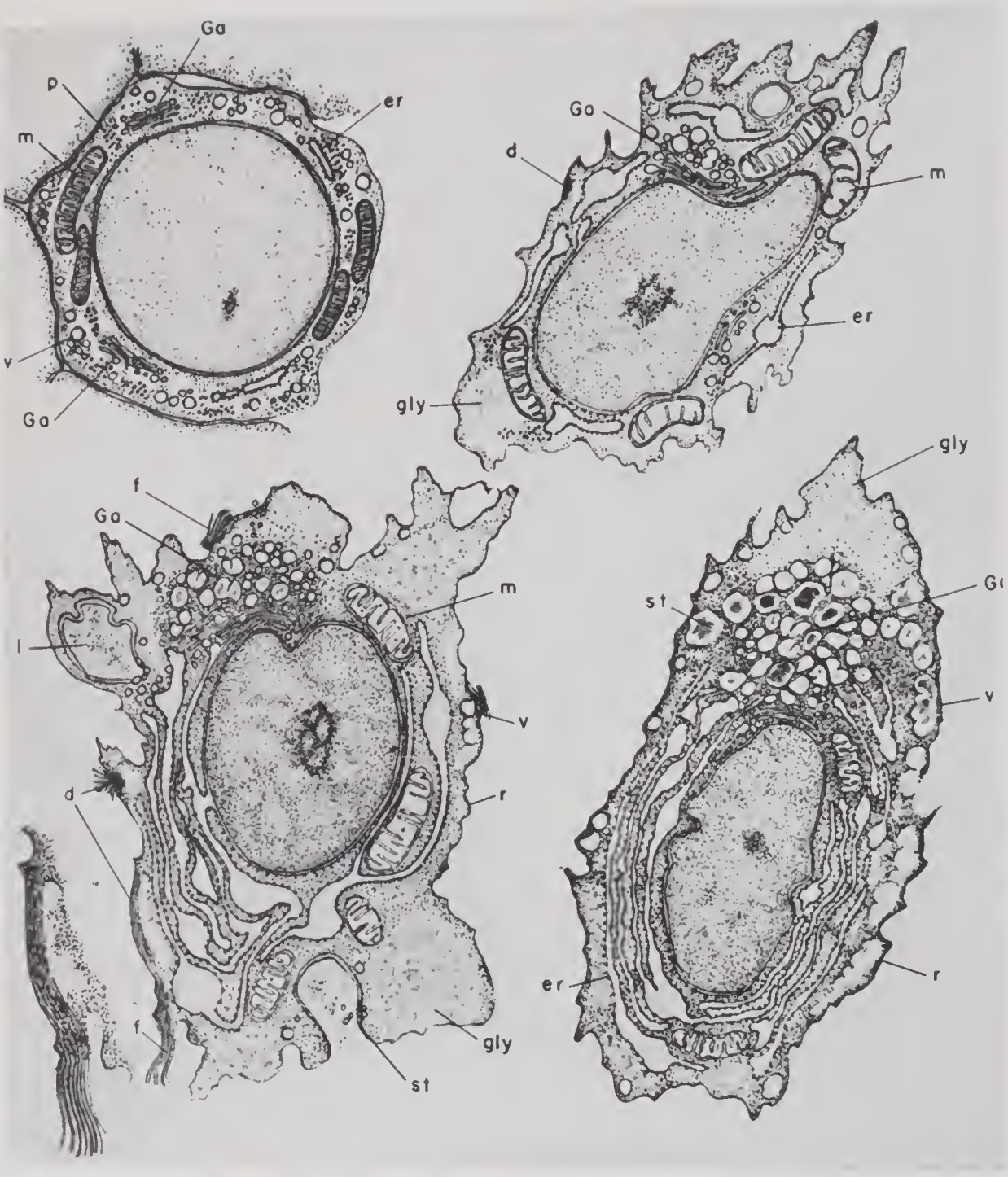


FIG. 36. A schematic representation of the successive stages in the differentiation of the chondroblast from mesenchymal cell to chondrocyte, illustrating the development of cellular organelles during this process. Top, left: the prechondroblast; top, right: early chondroblast; bottom, left: fully developed, actively secreting chondroblast; bottom, right: early chondrocyte. Key: (v) smooth walled vesicles; (p) free ribonucleoprotein granules; (m) mitochondria; (er) endoplasmic reticulum; (Ga) Golgi areas; (gly) deposits of glycogen; (d) regions of increased density in the cortical cytoplasm; (r) vacuoles lodged at the cortex under the cell membrane where they may lose their membranes; (f) fibrils; (l) lake limited by a double membrane; (st) stoma involved in extrusion mechanisms. Courtesy of Godman and Porter (1960).

alteration in cytoplasmic organization. It is not yet clear, however, how such intracellular structures are derived; it is possible that increased cellular organization and changes to the synthesis of specialized substances by the cell are dependent on each other. The detailed study of chondrogenesis by Godman and Porter (1960) has demonstrated the increase in complexity of the cytoplasmic organization during cytodifferentiation (Fig. 36).

IV. The Formation of Connective Tissues

A. *Fibrogenesis*

1. *Historical*

In 1757 Albrecht von Heller introduced the term "textus cellulosus" to distinguish the "elementary substance of the body" and pointed out that this fibrous material was concerned with the construction of most of the organs in mammals. Bordeu (1767) further described the "textus cellulosus" as the "most extensive and useful cellular organ of all the parts of the body; it nourishes all the organs, it connects one with the other and favours and entertains their mutual relations." Wolf (1774) supported the existence of a secondary substance in which the fibers lay, i.e., the "mere glue" of von Heller, and also demonstrated the coexistence of "globules" which he compared with the "minute bodies" of Hooke (1665). At the same time Boerhaave (1787) made the surprising suggestion that the fibers originated from tissue juices; previously to this no definite suggestion had been made as to the possible origin of the fibers. It was not until 1830, however, that Johannes Müller proposed that the name *Bindegewebe* (= connective tissue), should be applied to the white fibrous tissues.

Meanwhile, Purkinje (1825) had recorded the presence of a sphere —the nucleus—within animal globules and also had used the term protoplasm for the whole unit. The realization of the fundamental concept of cellular organization was then established by the investigations of Schleiden (1838) and Schwann (1839). They both claimed that the cells were the living units; the existence of the fibers of the connective tissues, however, could not be disregarded, especially in view of the long-held concept that the fibers formed the structural basis of the body. The relationship between the cells and these fibrous substances thus became a subject for endless discussion. The observations of Schleiden and Schwann were greatly enhanced by the development of the achromatic objective of Amici and Lister.

Schwann held that the fibers originated by a fibrillar division of the surface regions of the cells, which was followed by fusion of the individ-

ual fiber units with each other so as to form a somewhat granular mass of fibrous tissue; he thus favored a cellular origin for the fibers. Henle (1841) brought forward the proposal that cells, which he termed primordial cytoblastemas, arose from a blood-borne substance, i.e., lymph, and that subsequently connective tissue fibers developed from residual cytoblastomas after their breakdown. The discussion at this time involved not only the question of the origin of the fibers but, perhaps of more importance, the basic problem of whether the fibers when fully formed were cellular components or whether they were extracellular. Reichert (1845) pointed out in this connection that components of animal bodies that did not correspond to cells must be formed from part of the protoplasm or from the result of cellular action, and thus he introduced the idea of the existence of cell secretions. The issue became further confused when Virchow (1852) stated that life did not exist outside the cell, a statement from which it could be inferred that cell secretions, and fibers if they were extracellular, could no longer be considered as living matter.

It was generally recognized, however, that whatever the final form of the fibers might be, their formation depended on local cellular activity either by the fibers being extensions or transformations of the protoplasm, or by development of extracellular fibers as a result of secretion from the cells.

The first concept was very strongly supported by most of the classical histologists, although the literature of this time is most confusing, for there is often no clear distinction drawn between the possibilities that the fibers were intracellular in origin but extracellular in final form, or that they were direct transformations of protoplasm. Remak in 1852 claimed that the fibers were extensions or elongations of cells and that they formed a symplasm; De Bary (1859) demonstrated their structural continuity with the cells, and Schultze (1861) agreed that while the cells were continuous with each other the fibers were derivatives of protoplasm. These views became incorporated into the symplastic theory, in which it was stated that the properties of protoplasm were contained within the fibrous regions. The complete theory has been reviewed in detail by Cameron (1953).

At the same time, Beale (1861) agreed that though the fibers were formed at the periphery of the "germinal matter," once formed they no longer retained any vestige of life. On the other hand, Heitzmann (1873) succeeded in resolving a fine protoplasmic network which lay between the fibers; he suggested that this material was a ground substance and assumed that the presence of protoplasmic material demonstrated its living qualities; Strickler (1883) supported this view.

In 1871 Flemming suggested that a homogeneous material was present in specific cells associated with connective tissue, and Boll (1872) carried this hypothesis one stage further and postulated that the fibrous material was derived solely from one type of cell, the fibroblast. Retterer (1896) further supported the syncytial theory, and a year later Lukjenow (1897) reiterated that the fibers were products of protoplasmic transformation. Hansen (1899) then stated that the ground substance was homogeneous and cytoplasmic in origin; that the cytoplasm of cells differentiated into endoplasm and ectoplasm, the latter then expanding to form a syncytium of cells the meshes of which contained tissue fluid. Strict separation between fluid and ground substance, or hyaloplasm, was not possible, and he further postulated that the fibers were formed either within or at the interphase with the tissue fluid. Studnicka (1902) developed this idea further by suggesting that the ground substance finally became extracellular and that it was still capable of fiber formation when in that stage. Mall (1902) put forward a somewhat different structural point, viz., that the exoplasmic regions of the cell split off to form fibers while protoplasmic networks extending from the cells formed a ground substance; he also suggested that a true ground substance existed in cartilage and bone and was derived from a cell secretion.

The other concept prevalent during the second half of the nineteenth century was that the fibers were intercellular and were due to a cell secretion. Virchow (1858), while recognizing the proliferative power of connective tissue, held that the cells secreted a substance from which the fibers were derived, but that when formed the fibers were dead; their function was that of a packing material. Von Kölliker (1861) agreed that the origin of the fibers was a cellular secretion, but he disagreed with Virchow by believing that the fibers were alive though intercellular. Somewhat later von Ebner (1897) suggested that fiber formation might be the result of physical forces acting on a colloidal secretory product of the cells.

Thus by the beginning of the twentieth century most histologists agreed that in their final form the fibers of connective tissue were extracellular, but this included the reservation that the fibers might be extensions of cell processes. The question of the origin of the fibers, however, remained unanswered. The advent of the tissue culture technique provided a new method of approach to the problem of fibrogenesis, and numerous papers have been published; three main hypotheses became current for discussion and will be briefly mentioned.

First, it was held that collagen fibers originated within the cell or in association with the cell surface. Mallory (1903) succeeded in staining

with acid fuchsin fine fibrous structures which appeared to lie just within the inner surface of the cytoplasm. These fibers also followed the outline of the fibroblast and its processes, which extended far into the intercellular spaces. M. R. Lewis (1917) claimed that, in cultures of connective tissue from the fowl embryo, fibers arose from the surfaces of the cells; she also observed that similar fiber development took place in cultures grown in a saline medium; from her evidence it seems possible, however, that the fibers were in fact very long cytoplasmic processes.

Second, it has been held that collagen fibers develop extracellularly and involve the conversion of fibrin into collagen. Thus, Baitsell (1915) showed that in cultures of adult frog tissue, fibers, which he presumed to be collagenous, developed from the fibrin of blood plasma. This view was also supported by Nageotte and Guyon (1934), who stated that fibrinogen was either an essential precursor or acted as a source of protein for collagen production.

According to the third hypothesis, which was the most strongly supported, the fibers develop extracellularly but are not derived from fibrin, although the fibrin threads might form a support for the collagen fibers during their formation. For example, Maximow (1929) cultivated rabbit thymus by his "double coverslip" method; he showed that fibers always appeared in the neighborhood of cells, and he believed that they were formed by the precipitation of soluble substances in the medium. These fibers were reticular in that they stained by silver methods, but after 25–35 days' growth they became oriented into parallel lines, probably in response to a mechanical pull exerted by the migrating cells, and began to take the Mallory stain for collagen. From these results Maximow concluded that reticular fibers represented an early stage in collagen formation.

Most of the tissue culture investigations supported the view that collagen fibers developed in the intercellular spaces, but little attention was paid to the formation or function of the ground substance. In the past twenty years the high resolving power of the electron microscope, improvements in physical methods for investigating molecular properties and configuration, and advances in biochemical techniques for studying the extracellular constituents and mechanisms of their synthesis have all contributed greatly to providing a more coherent picture of the processes of fibrogenesis.

2. *Morphogenesis of Collagen Fibrils in Connective Tissues*

One main phase in the differentiation of connective tissues consists of the biosynthetic and cellular processes that are involved in specific

macromolecular production, and some of the steps concerned in this phase have been discussed in earlier sections of this chapter. The aggregation of various macromolecular units and their subsequent orientation into assemblies that form characteristic extracellular patterns represent a second distinct phase; the evidence on four sequential steps that constitute the main structural processes involved in the development of the collagen framework of the tissues will now be discussed in some detail.

a. Chain formation. Evidence is not available yet on the important structural problem of whether the three polypeptide chains of the collagen molecule are generated as single chains, which are then spontaneously aggregated into the unique organization of the three-chain macromolecule or whether generation of all three chains to form a cohesive unit occurs simultaneously. Nor is it known whether chains of short length, corresponding for instance to one period ($\sim$ 640 A.), are first formed and then subsequently aligned to make longer units, or whether the length of chains initially generated equals that of the tropocollagen monomer ($\sim$ 3000 A.). The recent work of Piez *et al.* (1963), however, indicates that the amino acid composition of at least one of the three chains is different to that of the other two (Table VI).

b. Site of initial aggregation of definitive collagen fibrils. Critical studies of the site at which initial aggregation of definitive collagen fibrils occurs have been made since the advent of the electron microscope, but even now the matter is controversial.

Porter and Vanamee (1949) and Porter (1951) studied fibrogenesis of intact tissue cultures of embryonic skin and suggested that a gelated layer just beneath the cell membrane became organized into collagen fibrils, recognizable by their axial periodicity, or that this gelated material passed through the cell surface as an exudate and became "polymerized" into definite fibrils of 200–300 A. diameter on the cell surface. The establishment of the thin-sectioning technique, however, provided another method of approach to the problem. Wyckoff suggested that collagen fibrils of 650 A. periodicity were produced along the surface of the cells, seemingly as a primary product of the fibroblasts of amniotic membranes of fowl embryos inoculated with influenza virus. On the other hand, in an investigation of sections of regenerating tendon of the rat, Wassermann (1954) described "primary" fibrils of irregular nodular structure 150–200 A. in diameter, of limited length, and with tapered ends that lay not only on the surface but within the marginal zone of the cytoplasm constituting a cortical zone of the cells which "was developed from the inside and then detached itself at the outside." In 1956 Wassermann and Kubota observed that groups of developing "microfibrils" were

enclosed within "the ramified body of the fibroblast" in connective tissue from 10-day-old chick embryos.

In an intitial study of the development of connective and skeletal tissue in chick embryos, Fitton Jackson (1954) noted numerous filamentous processes within osteoblasts of the periosteum. The absence of any visible demarcation between the cells and the developing periosteal bone suggested that the intracytoplasmic filaments might become extracellular and participate directly in the early stages of fibrogenesis; alternatively the filaments might be homologous with cytoplasmic membranes. Further studies, however, led Fitton Jackson (1956) to state quite clearly that a precise distinction between intracytoplasmic filaments and cytoplasmic membranes had not been achieved in the initial work.

The detailed investigations of the properties and fine structure of developing tendon and bone led Fitton Jackson (1955–1957a–c) to conclude, from observations of tissue sections cut transversely to the fibril axis, first, that small groups of fibrils formed within the cytoplasm and, second, that collagen fibrils arose outside the cell, often in close association with the cell surface, this second method of formation predominating as age increased (Fig. 37).

The evidence presented strongly supported the hypothesis that aggregation into fibrillar form occurred when cellular secretions came into contact with interstitial fluid. The transformation of collagen "precursors" into definitive fibrous form might thus be due solely to a change in environment, the interstitial fluid supplying a medium of appropriate ionic strength and pH. Aggregation of fibrillar units would occur mainly as a direct result of the extrusion of cellular secretions into the intercellular regions and reaction with the fluid. Since W. H. Lewis (1941) had pointed out that surrounding fluid may be introduced into the cell by pinocytosis, the cellular secretions might come also into contact with such fluid within vacuolar bodies in the cytoplasm, and after transformation would appear as an intracellular formation of fibrils. Fitton Jackson (1956) also pointed out that small groups of newly formed collagen fibrils may be introduced into the cell by phagacytosis and thus appear to have an intracellular origin.

Furthermore, it was suggested (Fitton Jackson, 1956) that the transformation of collagen precursors may not be due solely to a change in environment, but that the interstitial fluid might provide additional organic and/or enzymatic substances essential for the formation of the intercellular material. The above hypothesis does not necessarily preclude other processes that may be carried on simultaneously and may be di-

FIG. 37a. Electron micrograph of a section of 10-day embryo showing the early growth of tendon. The collagen fibrils are only about 100 A. in diameter and are arranged in small groups, all clearly outside the cell, but some are in intimate contact with the surface of the adjacent cell. (Fitton Jackson, 1956). Magnification: × 38,000.

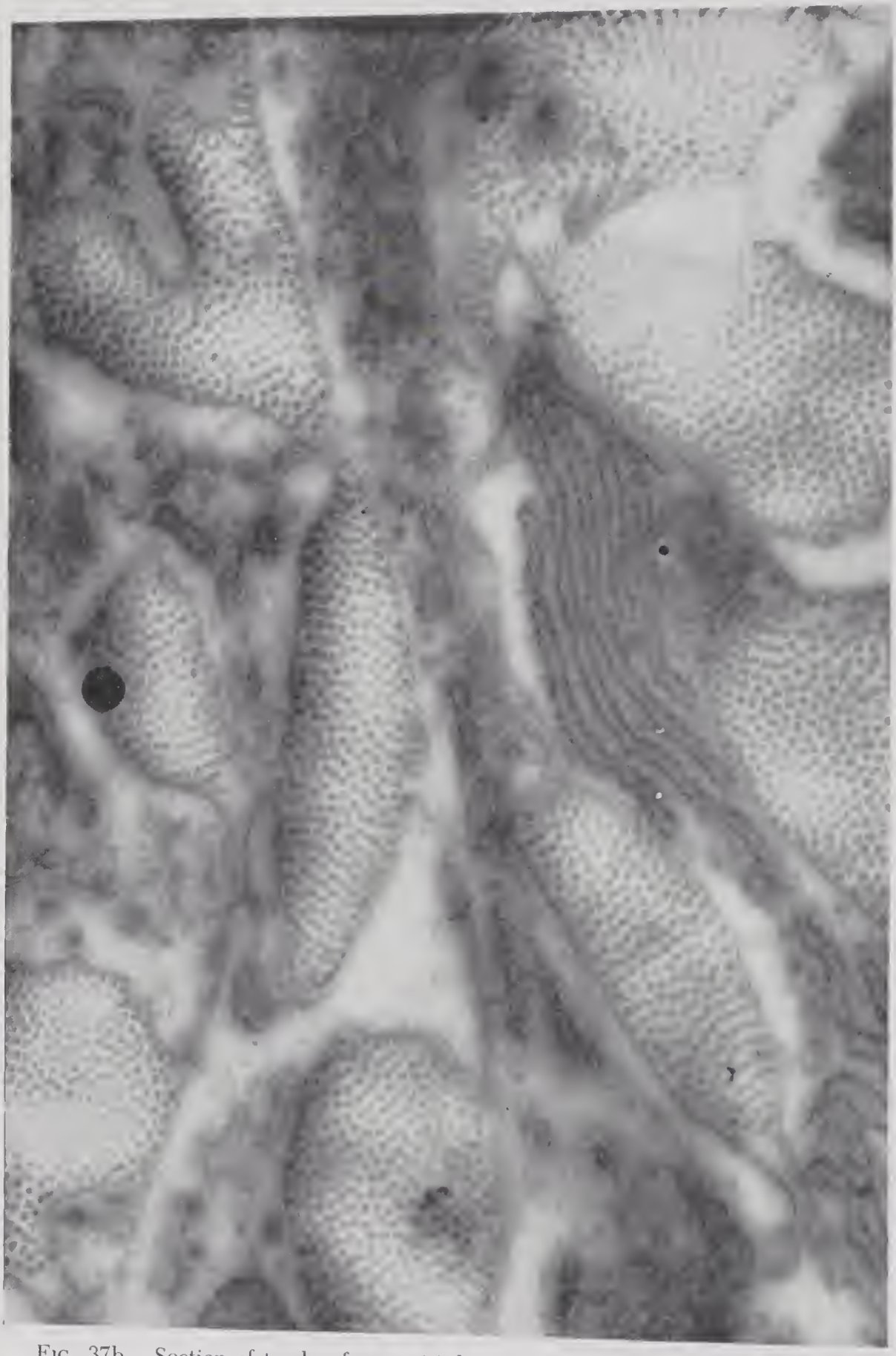


FIG. 37b. Section of tendon from a 14-day fowl embryo, showing how the intercellular spaces have increased in size and become filled with collagen fibrils; these areas have assumed the appearance of bundles and are closely interlocked with the cytoplasm of adjacent cells. The fibrils are about 250 A. in diameter and each one is invested by interfibrillar material (Fitton Jackson, 1956). Magnification: × 38,000.

rectly eoneerned with the control of both fibril formation and subsequent growth. These points will be discussed in detail below.

In a further detailed analysis of whole mounts of chick dermal tissue cultures viewed by electron microscopy, Porter and Pappas (1959) stressed that the appearance and amount of newly formed collagen fibrils bore a direct density relationship to the cell population and that fibrils were not present in the free medium at the periphery of the culture. Furthermore, they failed to reproduce the results of Doljanski and Roulet (1933), who had reported that collagen fibers, identified on histological criteria, developed on a porous glass filter, on the opposite side of which an explant was growing, thus suggesting that a cellular secretion diffused through the filter and interacted with the culture medium to form fibers.

Porter and Pappas noted that after about 48 hours in culture collagen fibrils eondensed at the surface of the cells, often in assoeiation with longitudinal thickenings of the cytoplasm. In thin sections of skin from 14–16-day chick embryos Porter and Pappas (1959) described areas of increased density at the cell surface which were often intimately asso-eiated with extracellular bundles of unit fibrils; other areas in which sueh fibril bundles lay close to the cell surfaee showed a normal density; and areas of increased density at the cell surface, but without closely asso-ciated bundles of unit fibrils. It was concluded from these observations that the unit fibrils of 200–300 A. diameter formed from material at the cell surface and were then shed into the extracellular regions, possibly by a meehanism similar to "ecdysis." Intraeellular fibrils were not ob-served. Porter and Pappas noted that in some circumstanees collagen fibrils might be able to form at a distance from the eell and that viewing the cultures *in situ* might enhance the apparent formation of eollagen fibrils at the eell surface.

This belief would be in agreement with observations by Fitton Jaek-son, who found in thin sections of osteoblast cultures, 48–72 hours old, cut at right angles to the plane of the culture where two layers of cells had formed, that intercellular collagen fibrils were situated close to the exterior surface of the cell as well as randomly disposed between and around the exterior surface of the osteoblasts for upward of 5 μ (Fig. 38). If such cultures were viewed *in situ*, the fibrils appeared to be closely associated with the surface of the cells, presumably owing to drying down on the speeimen grid. These findings implied that the fibrils were formed in close association with the eell surface and then moved into the extracellular area and/or that fibril formation may have oecurred also at some distance from the eell.

In detailed electron microscopic studies of wound healing in female

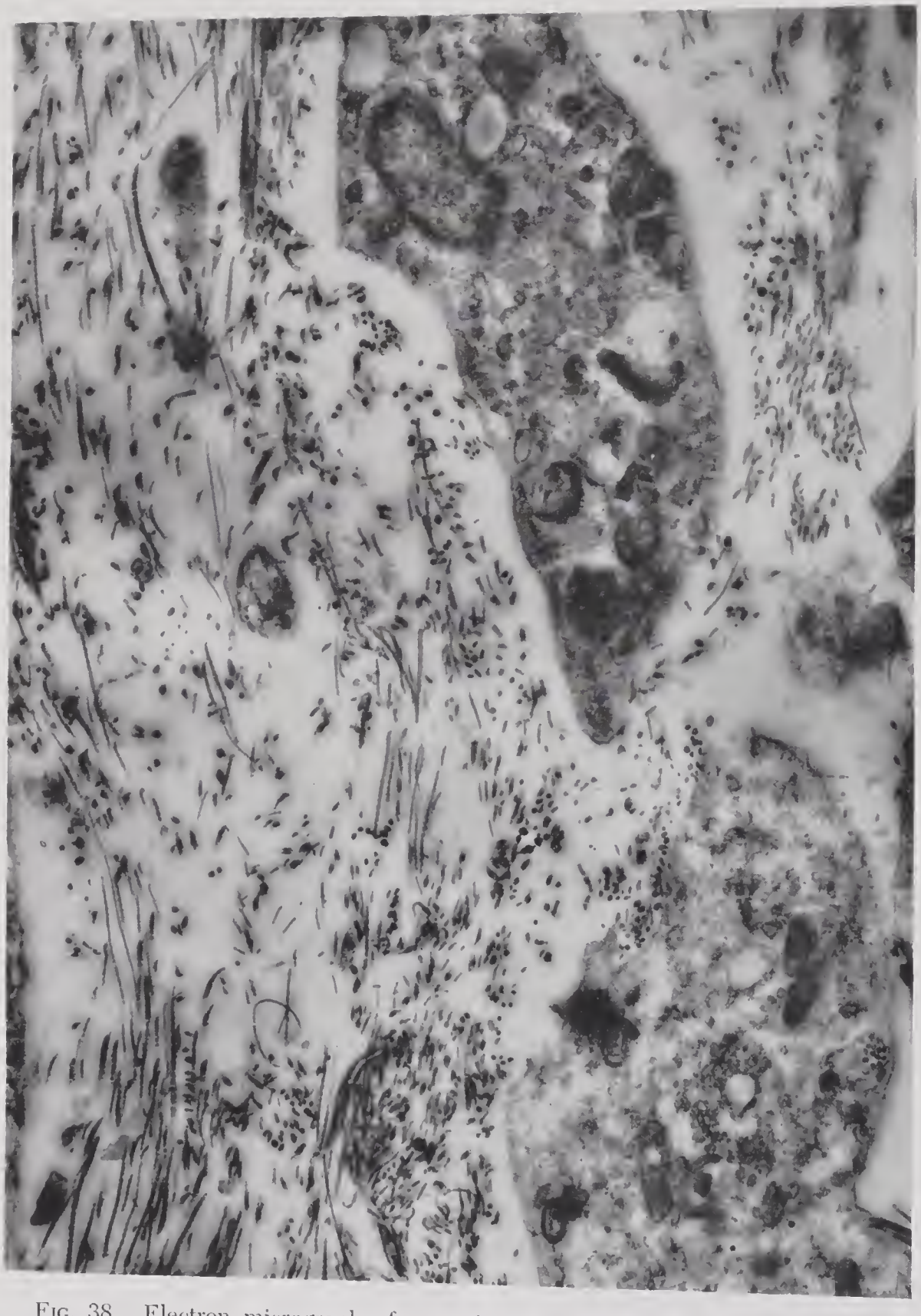


FIG. 38. Electron micrograph of a section of an osteoblast culture of 48-hour growth. Note the distribution of the collagen fibrils in relation to the cytoplasmic regions; it is clear that they are separated from each other and only a few are in close association with the cell surface. Magnification: × 22,000.

guinea pigs of 300–350 gm. weight, Ross and Benditt (1961) stressed that the cell surface membrane was clearly visible in all cells; they suggested that collagen fibril formation occurred by two methods after the secretion by the cells of a soluble precursor: first, by aggregation close to the cell surface at condensation points on the surface of the cells, i.e., extracellular formation dependent upon the cell; second, by "free" aggregation in the extracellular milieu. These authors also observed intracytoplasmic filaments, 20–80 A. in diameter, in parallel array near the periphery of some cells; it was not known, however, if the filaments were involved in the formation of collagen fibrils. Ross and Benditt calculated that a periodic structure would not be visible in filaments of less than 56 A. diameter so that the identification of thin filaments as collagenous by electron microscopy is not very feasible.

The studies discussed above uphold that the site of initial aggregation of definitive collagen fibrils occurs at, or in close association with, the cell surface and also at some distance from the cell. The state of molecular or macromolecular organization of the collagen units within the cell is not known; and whether any other substance, for example, polysaccharide or noncollagenous protein, is linked to part of the collagen configuration is open to question. The mechanism of extrusion of collagen from the cell must depend in part on the nature and the molecular weight of the product; it should be noted that in tissues with a comparatively high collagen content, such as in tendon and bone, globules characteristic of secretory cells have not been recorded close to the surface of the cell. Although the integrity of the unit membrane at the cell surface is a constant feature of fibrogenesis, in the studies discussed above the dynamic nature of the cell surface has been stressed (Fitton Jackson and Randall, 1956a); it seems possible that an enzyme, such as alkaline phosphatase, may be located at or just within the unit membrane of the cell surface and may mediate in an extrusion mechanism leading to the spontaneous transformation of collagen units into fibrils on the change in environment. It is interesting to note that although the diameter of the fibrils adjacent to the cells varies in different tissues, it is seldom less than 60 A. when seen in transverse section; this observation implies that about 14 tropocollagen units would be required to form the fibril if they were close packed in hexagonal array.

A number of investigations of fibrogenesis have been made, however, in which it was suggested that a breakdown occurred in the cell surface during extrusion of collagen; such observations were usually correlated with the appearance of thin filaments within the endoplasmic region of the cell. In preliminary observations by electron microscopy of sections

of tissue eultures grown from ehiek embryo heart, Yardley *et al.* (1960) and Yardley (1962) deseribed, in fibroblasts, marginal eondensations of the eell surface with related intracytoplasmic filaments, some of whieh appeared to be direetly associated with eharacteristie collagen fibrils in the extracellular phase. The mierographs indicated that during the process of fibrogenesis, the unit membrane of the cell surface disappeared or was disrupted within the regions of marginal condensations and that large eytoplasmie fragments ineluding filamentous material beeame detached from the main body of the fibroblast and were expelled into the extraeellular regions; the implication was made that the filaments were collagenous. Yardley *et al.* compared their observations with the appearance of extrusion of cellular material from apocrine cells; they suggested that transfer of material eould oecur also through the intaet cell membrane. That the disintegration and dissolution of peripheral cytoplasmic zones oeeurred during the fibrogenetie proeess was concluded also by Gieseking (1960). Further support for this view eame from electron mieroseopie study of granulomata indueed by earrageenin; Chapman (1961) reeorded the presence of cytoplasmic filaments, about 50 A. in diameter, eoncentrated in the outer region of the cytoplasm of the fibroblasts and held that dissolution of the unit membrane at the eell surface led to the eoneomitant release of the filamentous eontent and eytoplasmie material, again implying that the eortieal filaments were eollagenous. Chapman concurred with the idea that fibrogenesis might be akin to the apoerine seeretory proeess.

The question arises whether the apparent disintegration of the cell surfaee and extrusion of cytoplasmie material and assoeiated filaments is a feature of fibrogenesis. The mierographs that have been shown appear to uphold this hypothesis; before such an interpretation of events is made, however, two points other than preparative eonsiderations may be of significance. First, many of the micrographs in whieh the delineation of the unit membrane is unelear show cytoplasmie processes and filaments in longitudinal seetion; in these mierographs the filaments oftcn appear to be "sprouting out" from the eortieal zone of the cell. Sinee the seetions are considerably thieker than the filaments, it is impossible to assess accurately the spatial relationship between filaments, collagen fibrils and the indistinct cell surface. The clarity of the unit membrane representing part of the cell surface depends on the plane of the section; when eollagen fibrils are seen in transverse section immediately adjacent to the eell a unit membrane is normally visible at all stages of fibrogenesis. Second, it should be considered whether the cytoplasmic material, observed in the extracellular regions may be related to degradation of the tissue

rather than to fibrogenetic processes. Chapman (1961) makes the point that other cells than fibroblasts do not show signs of disintegration and, therefore, that the disruption and breakdown of the surface of the fibroblast is not an artifact; cell death occurs markedly in both granulomata and tissue cultures of some age and may be more prevalent in fibroblasts than in other cell types, which would not therefore show signs of degeneration. It is significant that in the studies of *in vivo* material discussed previously, e.g., tendon, bone, skin, and wound healing, such cellular debris in the extracellular phase was not recorded.

c. The critical diameter of the fibril. It is apparent that the aggregation of collagen units into fibrillar form need not occur immediately on their extrusion from the cell, since individual collagen fibrils, which are already clearly outside the cell, increase in diameter with age subsequently to their initial formation. Most investigators have postulated that the collagen fibril increases in size by the accretion of further collagen molecules onto the peripheral surface of the fibrils, rather than by the aggregation of adjacent fibrils. The final size of the collagen fibrils in any one tissue is characteristic for that tissue but varies markedly in different tissues. Evidence obtained from a study of the formation of tendon fibrils by Fitton Jackson (1956) has shown that the initial collagen fibrils of any one bundle must have been formed almost simultaneously or by a very strictly controlled mechanism since the variation in fibril diameter was minimal; that, as the diameter of the fibril increased with age, there was a relative reduction in the interfibrillar material which surrounded each individual fibril and, that the young developing tendon gave rise to a characteristic X-ray fiber diagram for collagen. Thus, during the development of the tendon the additional macromolecules must be laid down on the collagen fibrils in the form and packing appropriate to the fiber pattern (Fig. 39).

To enable such regulated growth to occur, there must be a mechanism that permits the free passage of macromolecular units through the interfibrillar regions until conditions are appropriate and permit aggregation onto specific fibrils. Randall *et al.* (1955) pointed out that the electric charge and its distribution on the molecule are primary factors in the phenomena of fibril formation in connective tissues. Thus, when a collagen macromolecule or smaller unit passes through the interfibrillar milieu, the electric charges on the unit and on the neighboring fibrils must be in negative balance and aggregation cannot occur, but when the positive and negative charges on the molecular unit match those of an adjacent fibril, aggregation occurs. Such a mechanism implies that a substance within the interfibrillar material immediately surrounding certain fibrils

acts as a temporary barrier, or inhibitor, to molecular aggregation; Robertson *et al.* (1941) suggested that such a substance might be contained within the mucopolysaccharide moiety of the ground substance, but other components might be concerned also. If a substance were to react in this way, an agent would be required to alter conditions around individual fibrils appropriately before further accretion of collagen units could take place; it is possible that an enzyme may be concerned in such a mechanism that would control the rate of fibril growth. This hypothesis

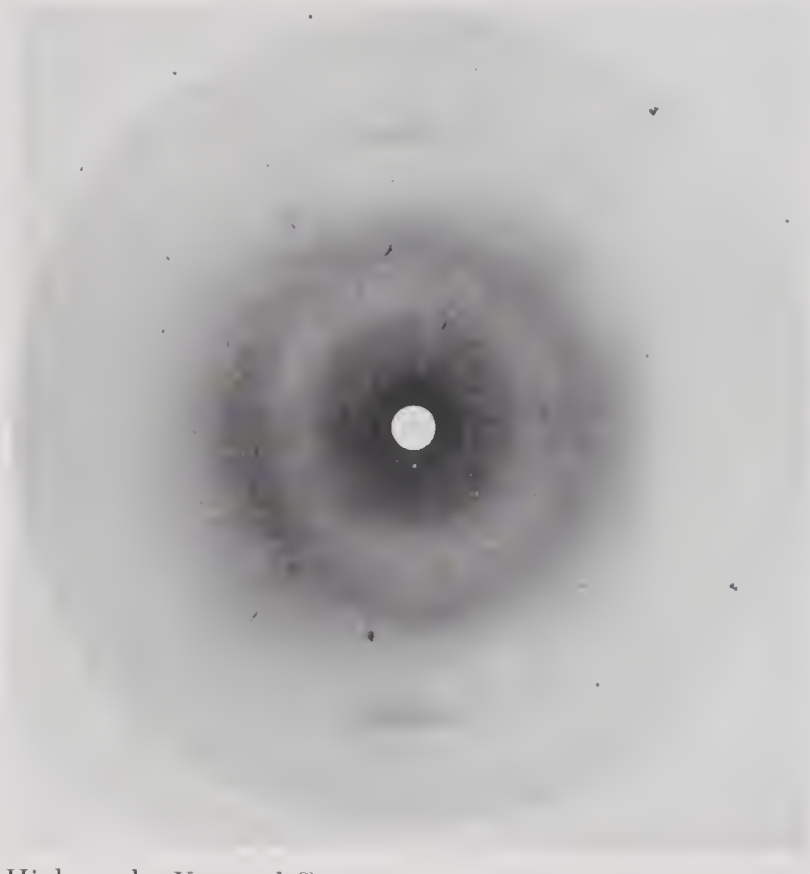


FIG. 39. High-angle X-ray diffraction pattern of tendon from an 11-day fowl embryo. The 2.86 A. reflection is apparent, thus indicating that even at this young age macromolecular orientation has occurred.

indicates a way in which a precisely balanced series of events could be achieved, and, as pointed out by Fitton Jackson (1956), the environment of the individual fibrils within a bundle at any one time during fibrogenesis would be of fundamental importance.

In this discussion it is necessary to recall that purified solutions of collagen are capable of being reprecipitated as characteristic collagen fibrils *in vitro* by, for example, simple alteration of the ionic concentration of the solution, and neither enzymes nor mucopolysaccharides are needed in this process. In fact, if mucopolysaccharides are added to collagen solutions in amounts greater than about 0.1%, the resultant pre-

cipitate is composed of fibrils with marked variation in periodic structure and are unlike those found *in vivo*; thus *in vitro* a far smaller amount of mucopolysaccharide than that present in most connective tissues disrupts the spontaneous aggregation of collagen units to form into characteristic fibrils. Furthermore, it is possible that the collagen units newly synthesized by the cell may differ in some respects from the macromolecular particles obtained from extracts of fully formed tissues, and hence aggregation phenomena may differ in the two systems.

The factors involved in the regulation of the characteristic diameter of the collagen fibrils in any one tissue are not known; nor it is understood what may influence the rate of synthesis of the various products of the extracellular phase that appear to be extruded from the cell either concomitantly or in alternating waves. The above discussion indicates, however, that a precise interrelationship must exist between the various constituents, and its supports the contention that a complex series of controlled events occur during the enlargement of the collagen fibrils.

d. Fibril orientation to form tissue fabrics. The spatial organization of the cells in many tissues is such that they appear to have had a general influence on the large-scale structure of the respective tissue. The orientation of the collagen fibrils within each tissue is usually precise, the pattern often being one of single or multiple layers of fibrils arranged at certain angles with respect to each other. A classical example is that of the cuticle of the earthworm; electron microscope studies by Reed and Rudall (1948) showed that it consisted of single layers of collagen fibrils embedded in an amorphous material, each layer oriented at 90 degrees to the previous one. The developmental problems involved in such fibril orientation are not yet understood, but it is conceivable that some form of macrocrystallographic order exists in connective tissue (Weiss, 1957).

A number of studies in support of this concept have been made of the organization of basement lamella of amphibian larvae. Collagen fibrils about 500 A. in diameter are formed into an orthogonal layered pattern immediately beneath the epidermis (Fig. 40). It is interesting that Edds (1958) noted that bound hydroxyproline was not detected until a thin layer of characteristic collagen fibrils appeared beneath the epidermis. More recently Edds and Sweeny (1961) have suggested that new fibrils are added to the lamella at its epidermal surface, thus implying that epidermal cells may contribute necessary products for the development; Hay and Revel (1963) have shown that tritiated proline is localized within both dermal fibroblasts and epidermal cells and that it accumulates at the epidermal-lamellar junction (Fig. 41), a finding again sug-

gestive of the concept that the epidermal cell is intimately concerned in the formation of the basement lamella.

Studies of the sequence of events that occurred during the development of the basement lamella in urodeles led Weiss and Ferris (1956) to suggest that the structural order must have been achieved by the regrouping of units of lower but distinctly supramolecular order, the fibril orientation possibly being determined by an underlying lattice system present in the ground substance. Alternatively, the last fibril layer

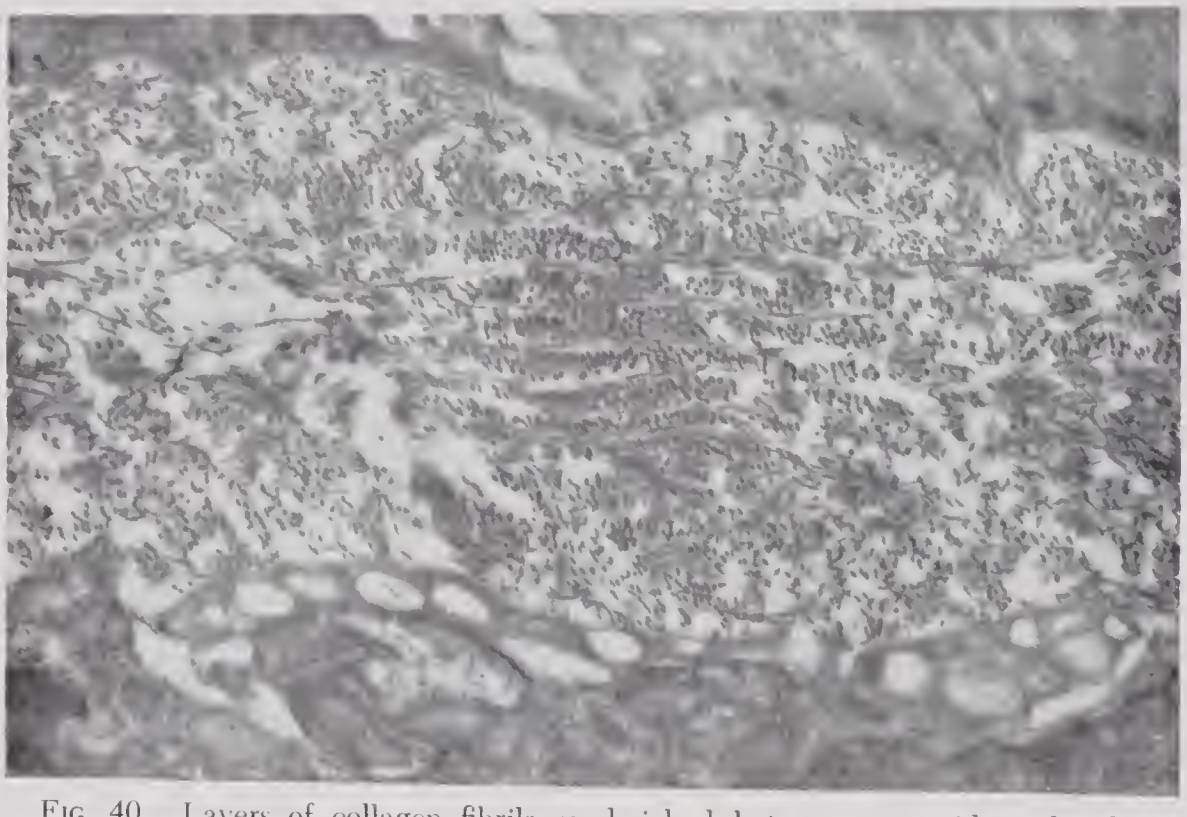


FIG. 40. Layers of collagen fibrils sandwiched between an epidermal cell (at top) and a cytoplasmic extension of a fibroblast. Electron micrograph of the skin of *Amblystoma* larva. Magnification: × 25,000.

of one ply might impose the rectangular orientation upon the first layer of the adjacent ply. In urodeles about 5 layers of collagen fibrils were contained in each ply, whereas in anurans each ply consisted of a single layer of fibrils (Kemp, 1959). Weiss and Ferris (1956) suggested that there must be a certain condition of equilibrium in the tissue that controlled the precise depth of each layer of collagen and the consistent total thickness of the basement lamella. A further instance of the ability of collagen to form into precise organized arrays is illustrated in Fig. 42.

B. Chondrogenesis

Although cartilage cells are essentially modulated forms of the same stem cell common to all connective and skeletal tissues, the proportions

FIG. 41. Electron micrograph of an autoradiograph of the epidermal-lamellar junction in *Amblystoma* limb fixed 4 hours after administration of H^3-proline. Note that the labeled material is predominantly extracellular. Courtesy of Hay and Revel (1963). Magnification: × 16,000.

of the various constituents produced by them differ markedly from those produced by cells of most of the other connective tissues. One of the chief characteristics of cartilage is a resilient matrix attributed to the high concentration of the proteinpolysaccharide complex present in the matrix and which may constitute as much as half the dry weight of the

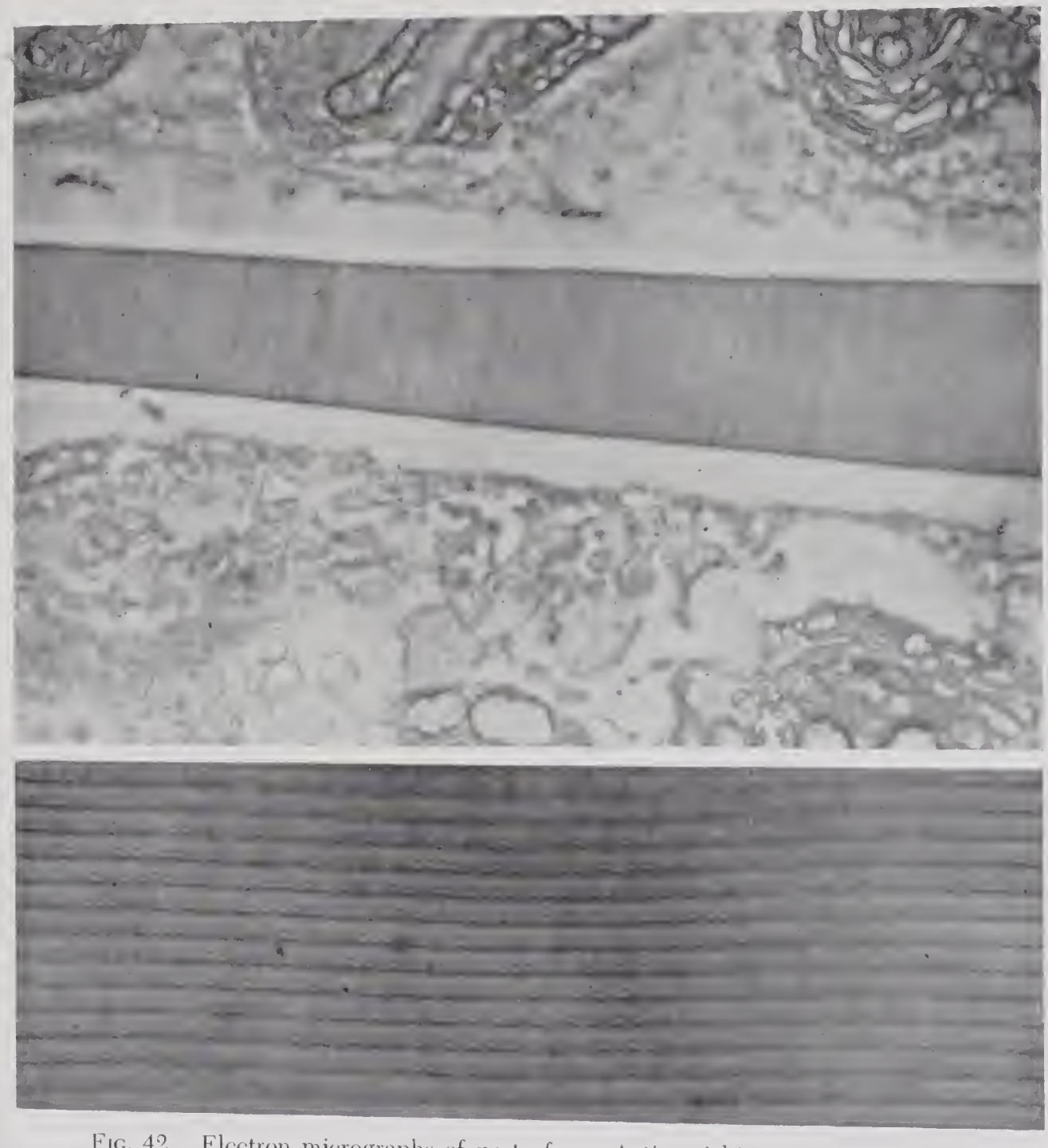


FIG. 42. Electron micrographs of part of one Actinotrichia present in the regenerating tip of a fish tail. These rods are composed of elastoidin; the top picture indicates the precise organization of the rod, while the bottom micrograph shows part of the banded structure demonstrating the specific alignment of adjacent macromolecules. Magnification: (top) × 8000; (bottom) × 55,000.

tissue. The question arises, therefore, whether the high proportion of this substance affects the manner of extrusion of the specialized products from the cells and the basic processes concerned in the formation and development of the collagen fibrils.

1. The Morphogenesis of Cartilage Matrix

In an elegant study of chondrogenesis by electron microscopy Godman and Porter (1960) and Godman (1962) described the progressive stages of differentiation in epiphyseal cartilage of fetal rats and suggested that specialized areas of the cytoplasm of chondrogenic cells were involved in matrix formation. Three morphologically distinct zones were identified. The first was an ectoplasmic region of the cell in which unit or primary fibrils 70–100 A. in diameter were present; it was suggested that these collagenous unit fibrils were continually dispersed in groups into the intercellular regions where they increased about three times in width, presumably by the addition of monomeric units of tropocollagen secreted by the cells. In the second zone, vacuoles of various sizes, delimited by a single membrane and containing a substance of moderate density, were associated with the Golgi zone of the cell; with differentiation the vacuoles enlarged and appeared to be discharged from the cell either as whole microvesicles or by coalescence with the surface of the cell (Fig. 43). In the third zone, large lakes or pools, surrounded by a double membrane were present in the cytoplasm and contained very fine fibrillar and amorphous material, as well as small granules. The origin and content of these lakes were unknown, but they appeared to be expelled from the cell either by the formation of a bleb or by coalescence with the cell surface. In addition Godman and Porter noted that the accumulation of glycogen occurred during the hypertrophy of the cell, and this was interpreted as a process of senescence, that led ultimately to cell death, and the resultant contribution of cellular inclusions to the cartilage matrix.

Revel and Hay (1963), however, held that collagen was removed from the chondroblasts during chondrogenesis by membrane-bounded secretory vacuoles originating in the Golgi zone of the cell, and was not extruded via the cell surface as a fibrillar product of the cytoplasm. Revel and Hay made a combined electron microscope and autoradiographic study of the development of cartilage in regenerating limbs of *Amblystoma*; tritiated proline was administered intraperitoneally as a 10-minute pulse. The label was observed 15 minutes later in the cytoplasm of the chondroblasts of the regenerates, frequently in association with ergastoplasm. After about 20 minutes the labeled material began to

appear in the Golgi zone, and after 30 minutes it was mostly concentrated in this region. The product in the vacuolar bodies of the Golgi zone appeared morphologically similar to the amorphous matrix. Other micrographs showed vacuoles close to or in association with the cell surface, and some of them contained labeled material. These observations led Revel and Hay to suggest that the vacuoles originated in the Golgi zone

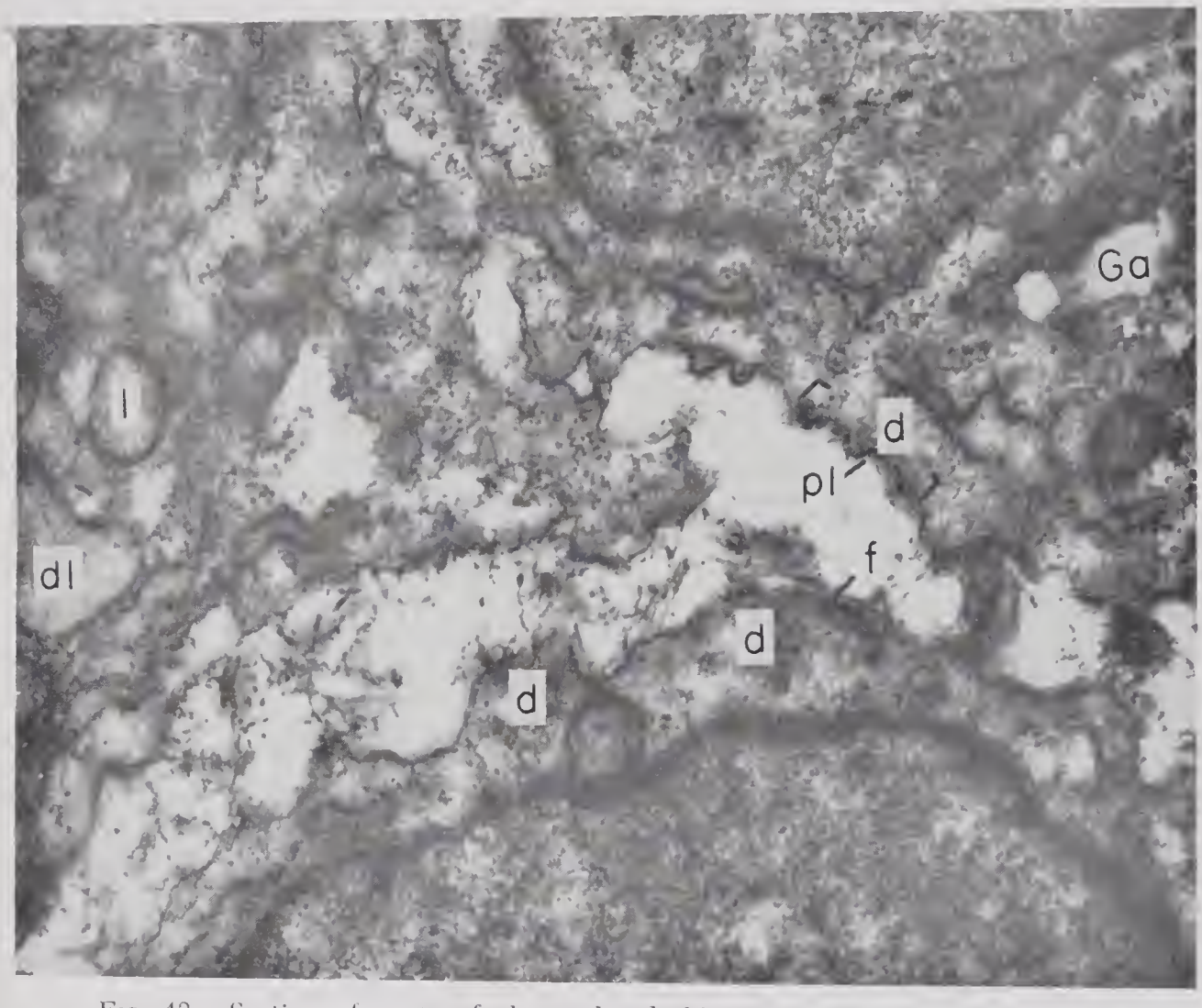
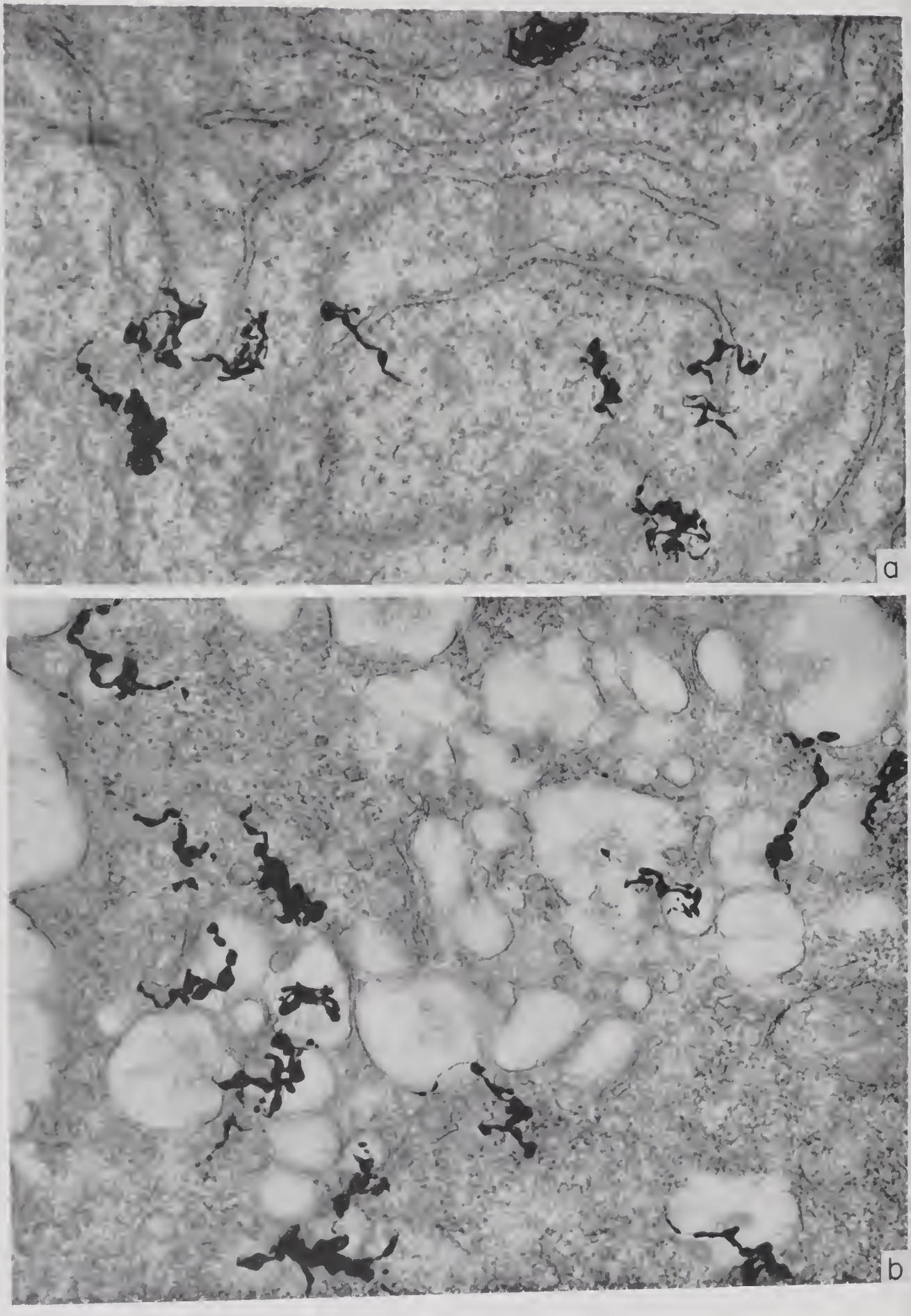


FIG. 43. Section of parts of three chondroblasts. Key: (Ga) extensive Golgi apparatus; (d) dense areas or bands of the cortical ectoplasm subjacent to the cell membrane; (f) sheath of parallel fibers at the cell surface; (dl) dilated cisternae of the endoplasmic reticulum; (l) small pool limited by a double membrane. Courtesy of Godman and Porter (1960). Magnification: × 30,000.

and then moved toward the cell surface membrane, where fusion took place and led to the extrusion of the labeled contents (Fig. 44). One to two hours after the administration of the isotope the labeled material was found predominantly in the extracellular phase; it did not accumulate adjacent to the chondroblasts, but diffused away from the surface of the cells into the cartilage matrix and began to form a band of isotopic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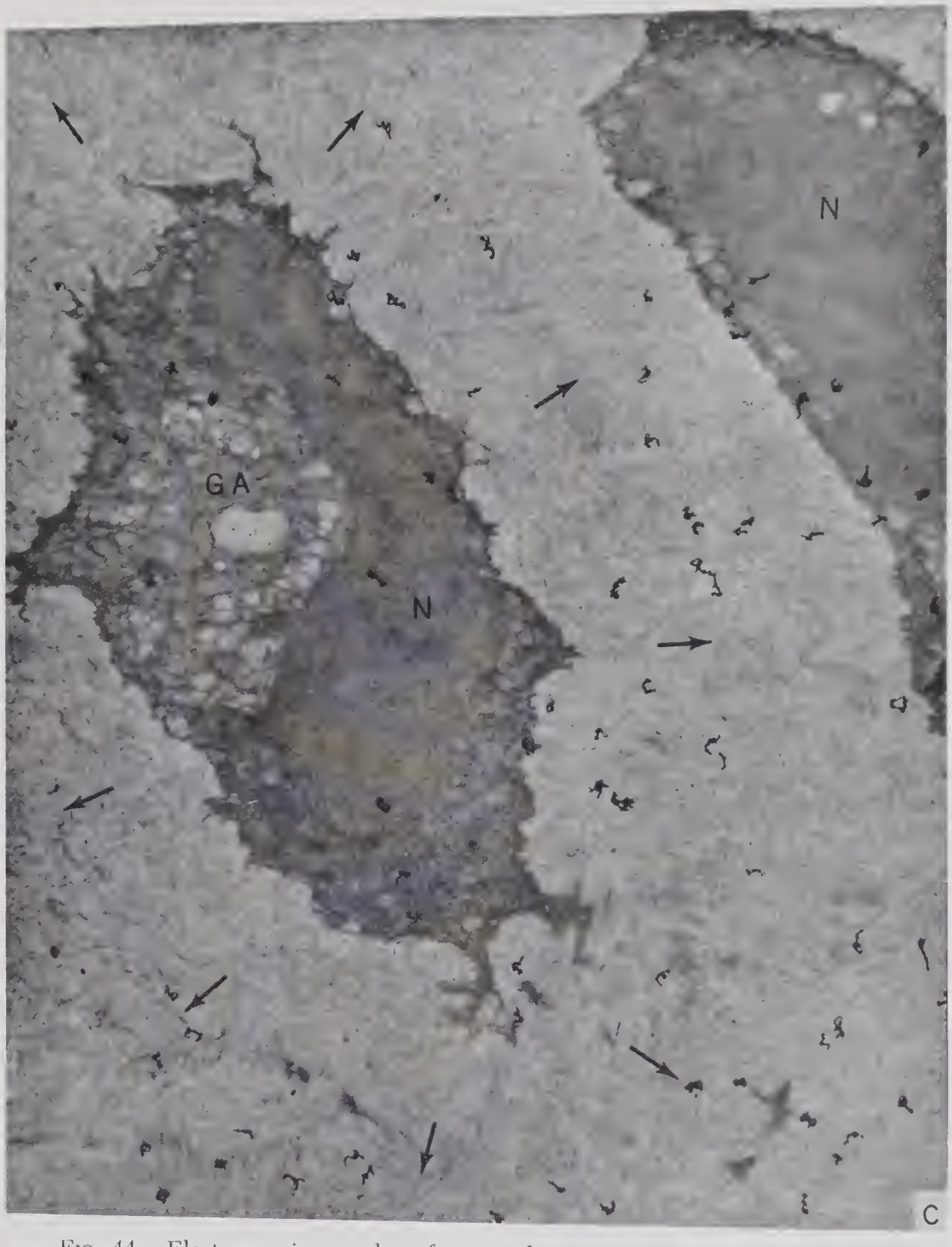


FIG. 44. Electron micrographs of autoradiographs of regenerating limbs of *Amblystoma*. (a) Portion of the ergastoplasm 15 minutes after administration of H^3-proline; many of the grains are localized in association with the cisternae. Magnification: × 33,000. (b) The Golgi complex 30 minutes after injection of the isotope; some of the grains appear to be contained within the vacuoles of the Golgi zone. Magnification: × 35,000. (c) General view of the cartilage 2 hours after the animal received the isotope. Many silver grains are located in the matrix and appear to be accumulating at some distance from the cells (arrows). Key: (N) nucleus; (GA) Golgi apparatus; lead hydroxide stain. Magnification: × 7000. All three micrographs are by courtesy of Revel and Hay (1963).

material localized approximately equidistant from the surrounding cells. Twenty-four hours later the labeled material had become concentrated in this position, and it was still present in the matrix 16 days later. Revel and Hay assumed that the tritiated proline represented collagen and suggested that it was synthesized in association with the ergastoplasm and then moved via the vesicles of the Golgi zone into the extracellular phase, as outlined above. In the view of the present author, however, the labeled material visualized in autoradiographs cannot be attributed solely to the collagen moiety of the matrix; part of the tritiated proline may have been incorporated, for instance, into the noncollagenous protein fraction of the proteinpolysaccharide complex of cartilage (see Table IV). Be this as it may, the timed sequence of events built up from the localization of isotopic material in regenerates by Revel and Hay supports the concept of an extrusion of secretory vesicles from chondrogenic cells during the development of cartilage matrix.

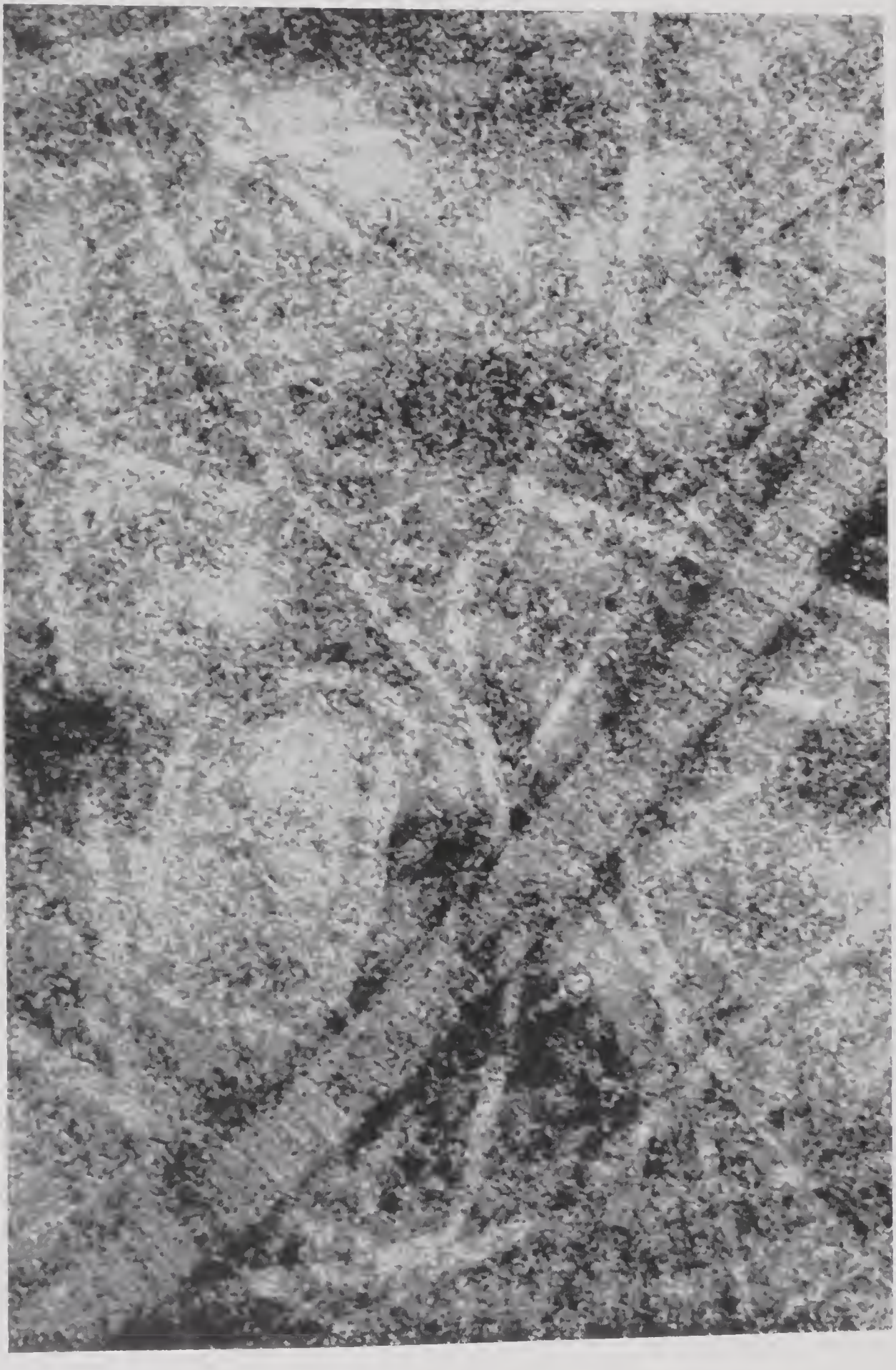
Thin sections of the matrix of most cartilages viewed by electron microscopy fail to show any clearly defined structural features; the extracellular phase appears essentially as an amorphous mass of low electron density, in which thin filaments, often about 200–300 A. in diameter are randomly disposed in relation to each other. Although various types of periodic structure are apparent in the fibrils, none are characteristic for collagen, but it has been generally assumed that the fibrils are collagenous, whereas the amorphous material represents the various constituents of the ground substance (Fig. 45). As mentioned above, Godman and Porter (1960) noted that, with age, the diameter of the fibrils increased to about 300 A. Revel and Hay (1963) found that whereas fibrils close to the cells were not more than 100 A. in diameter those farther away in the matrix varied between 300 and 400 A.; this observation could imply that collagen fibrils were formed near the cell as new matrix was laid down, and enlarged as the amount of matrix increased. Collagen fibrils of articular cartilage show a characteristic periodicity and are at least 600 A. in diameter.

A study of the structure of cartilage matrix by electron microscopy and the negative staining technique by the author has demonstrated that a large proportion of the proteinpolysaccharide extracted from chicken cartilage contained long, particulate filaments (Figs. 7–10). Squash preparations from four regions of cartilage from avian long bones viewed by the same method were seen to consist essentially of characteristic collagen fibrils, thin filaments similar to those found in the proteinpolysaccharide moiety, and many particulate units randomly disposed which may represent different states of aggregation of the proteinpolysaccharide

(Fig. 46). Hence the apparent amorphous structure of the ground substance as seen in thin sections of cartilage by electron microscopy is misleading. Furthermore, are all the fibrils seen in thin sections collagen, or is it possible that some at least are filaments of proteinpolysaccharide?

FIG. 45. Section of cartilage matrix as seen by electron microscopy. The specimen is tibial cartilage of 5-day chick and shows the junction between the epiphyseal plate and the hypertrophic zone at the bottom of the picture. Part of the surface region of a chondroblast is shown at the top right-hand corner. Magnification: × 30,000.

In the negatively stained preparations the periodicity of the collagen fibril is often masked by associated proteinpolysaccharide; this may be due solely to a preparative artifact, but it is possible that *in vivo* an intimate interrelationship exists between ground substance and collagen and



leads to the lack of clarity in the periodic structure of collagen fibrils seen in sectioned material.

These investigations have shed new light on certain aspects of chondrogenesis. The detailed morphological study of rat epiphyseal cartilage by Godman and Porter (1960) has led to the suggestion that the ground substance is secreted from the chondroblasts by the extrusion of vesicles originating in the Golgi zone of the cell. This hypothesis would be in accord with the general behavior of secretory cells, in which newly synthesized products are condensed and packaged into globules and stored in the Golgi zone prior to their extrusion. That such a theory may be applied to the mechanisms involved in the production of cartilage matrix is upheld by studies with radioactive isotopes (Fig. 47a) (see pages 421 and 461). Whether all the various constituents of the ground substance synthesized by the chondrogenic cells pass through the Golgi zone is not known; nor is it clear what state of macromolecular aggregation has been reached by the proteinpolysaccharide moiety. A hypothetical scheme of the various steps involved in the aggregation of this material is shown in Fig. 14; it is evident from this figure that many steps must be concerned after the synthesis of the component molecules before the macromolecular configuration is completed. It may well be that aggregation of the proteinpolysaccharide macromolecules into filamentous units occurs outside the cell, the excess free Na^+ ions present in the matrix supplying the correct milieu. A point of considerable interest is the way in which a highly negatively charged substance is discharged from the cell; the localization of the substance within a membrane-bounded vesicle until it is itself extruded from the cell provides an ideal mechanism.

The site of initial collagen fibril formation in cartilage is still controversial. On the one hand, Godman and Porter (1960) suggest an ectoplasmic formation of primary fibrils, although it is difficult in the micrographs to evaluate the precise localization of the fibrils and whether they are subjacent to the unit membrane of the cell surface; the fibrils are shed subsequently by "excortication" into the extracellular domain to form cores on which further tropocollagen units accrue. On the other hand, Revel and Hay (1963) propose that soluble collagen is concentrated first in the vesicles of the Golgi zone of the cells and then ex-

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FIG. 46. A squash preparation of cartilage from a 5-day chick showing the proteinpolysaccharide filaments and part of a collagen fibril as well as particulate units of proteinpolysaccharide randomly disposed. Negative-stained preparation. Magnification: × 140,000.

truded into the intercellular regions where fibril formation takes place; they failed to observe any ectoplasmic fibrils in their material. If collagen is represented, at least in part, by the tritiated proline, the Golgi zone may be concerned with more than one major constituent of the extracellular materials. If this is true, it would seem probable that individual Golgi vesicles would contain only one distinct substance at any one time rather than a mixture of macromolecular species.

The evidence available indicates that the diameter of collagen fibrils in, for example, epiphyseal cartilage is small in comparison to that in many other tissues; it is interesting that there appears to be an inverse relationship between the diameter of collagen fibrils and the amount of ground substance present in the respective tissue. Do any constituents within the ground substance determine the ultimate size of the collagen fibrils, and/or do the synthetic mechanism in the cell respond to environmental conditions so that the correct proportions of the different constituents are maintained? Recent experiments by the author have shown that when solutions of collagen and proteinpolysaccharide fractions obtained from developing chicken cartilage are mixed in amounts approximating those found in the tissue, the optical density of the solution increases, but characteristic collagen fibrils are not present; the proportion of proteinpolysaccharide must be greatly reduced before typical fibrils are formed. This finding indicates that in chondrogenesis additional factors must react to permit the orderly alignment of collagen macromolecules into fibrillar form in the presence of large amounts of proteinpolysaccharides.

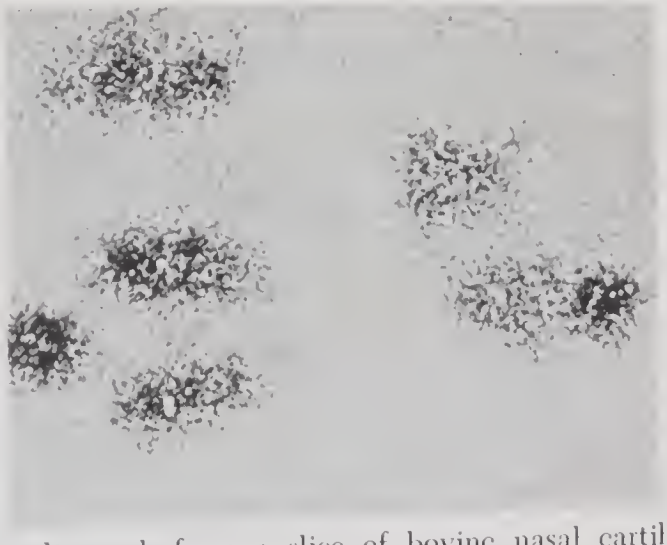
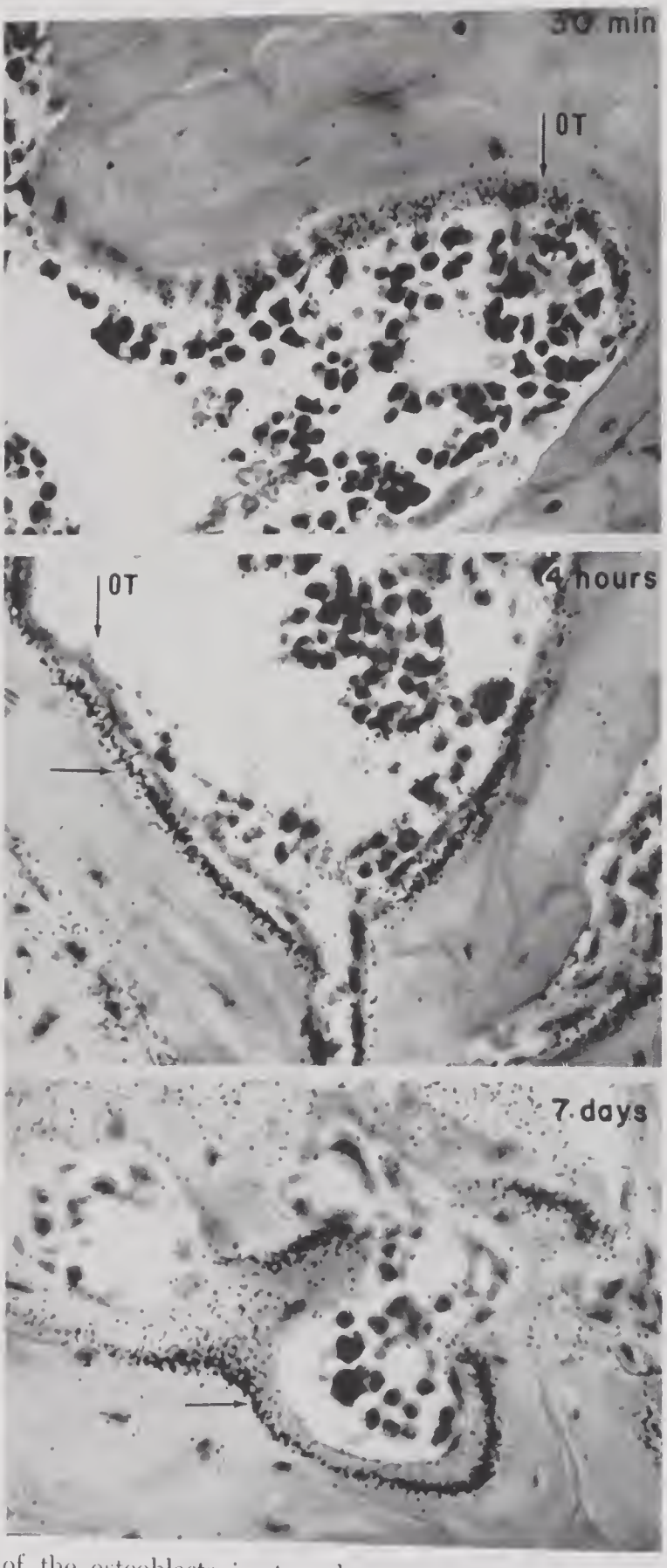


FIG. 47a. Autoradiograph from a slice of bovine nasal cartilage incubated for 2 hours in buffered solution of salt containing S^{35}-sulfate. The tracer is localized predominantly in the chondroblasts. Courtesy of Campo and Dziewiatkowski (1962).

FIG. 47b–d. Hematoxylin-eosin stained autoradiographs of growth zones in tibia or femur of glycine-H^3 injected into mice. (b) Thirty minutes after injection



the cytoplasm of the osteoblasts is strongly reactive. (c) After 4 hours the radioactivity of the osteoblasts decreases, but prebone becomes heavily labeled (arrow). (d) Reaction band is clearly seen 7 days after administration of the isotope. Courtesy of Carneiro and Leblond (1959).

C. The Process of Early Mineralization

1. Ossification of Periosteal Bone

Robinson and his associates, by means of electron microscopy, made an extensive study of mineralization in mammalian bone (e.g., Robinson and Watson, 1952, 1955; Sheldon and Robinson, 1957). They noted that, shortly after the organic matrix had been laid down by the cells, an amorphous substance tended to mask the newly formed tissue; this phenomenon was called the calcification front. X-ray diffraction of de-

FIG. 48. Collagen fibrils of periosteal bone from 16-day fowl embryo showing the localization of the dense particles in relation to the structure of the fibrils. At the right a transverse section of one fibril at the appropriate level showing the arrangement of the particles (Fitton Jackson, 1957a). Magnification: × 100,000.

veloping bone demonstrated that the hydroxyapatite crystals that constitute the bone mineral became preferentially oriented with their *c*-axis parallel to the axis of the collagen fiber (Engstrom and Zetterstrom, 1951). Studies of the cellular processes involved in osteogenesis and the initial precise relationship between collagen fibrils and the mineral phase in periosteal bone of chicken embryos (Fitton Jackson and Randall, 1956a, b; Fitton Jackson, 1957a), showed that, when a small partition of bone matrix developed between two cells in which collagen fibrils could be clearly distinguished, dense particles less than 100 A. in diameter were precisely localized between the *d* and the *ab* bands of each period of the collagen fibril (Fig. 48; see also Fig. 28). Since in some of the

micrographs the fibrils must be seen in longitudinal section, it followed that the partieles were not only on the surfaee, but were embedded within the fibrils in an organized fashion. The diameter of these collagen fibrils was about 600–700 A.; in appropriate transverse sections the partieles appeared to be irregular in outline, were arranged in a ring about 100 A. apart from each other, and numbered up to ten (Fig. 48). Eleetron diffraction identified the presence of an apatite, but there was no preferred orientation of the partieles relative to the fibril axis at this stage. Fitton Jaekson and Randall diseussed whether the precise localization of the partieles was due solely to meehanical faetors, i.e., within the major regions of indentation of the period, or whether specific chemieal groups in the interband region were involved. Furthermore, Fitton Jackson pointed out that since the particles penetrated into the collagen fibrils to some extent, they might in turn distort the polypeptide chains in the interband regions. Such particles have been noted by, for example, Sheldon and Robinson (1957) in assoeiation with eollagen fibrils of young mouse bone and by Dudley and Spiro (1961) in human woven bone. Fitton Jaekson and Randall (1956a) noted that the opaque substanee assoeiated with an early stage in mineralization usually appeared at first some distanee from the cells and masked the fine strueture of the collagen fibrils. Although eleetron diffraetion confirmed the presenee of an apatite in areas where there was a preferred orientation of the collagen fibrils, there was no evidenee of arcing in the powder diffraction patterns; this finding suggested that the apatite might still be in an amorphous state (Fitton Jackson, 1957a).

2. Calcification in Cartilage

Such an arrangement of particles in relation to eollagen fibrils has not been observed in ealcifying cartilage of the long bones of chickens, nor, to the author's knowledge, has this assoeiation been noted in cartilages of other species. One study has shown that the initial steps involved in the calcification of cartilage may be followed precisely in the cell layers of the hypertrophic zone immediately adjaeent to the epiphyseal plate in 5-day-old ehicken tibias (Fitton Jaekson, 1960). As the chondrocytes, which at a slightly earlier stage had belonged to the lower layers of the flattened eells of the epiphyseal plate, began to hypertrophy, the surrounding matrix beeame strongly honeycombed. An amorphous material, with a eonsisteney different to that of the ground substance of the cartilage matrix, appeared within the eentral compartments at some little distance from the cells; this material contained a fine particulate substance as well as short needlelike particles, each about 150 A.

long. About two cell layers deeper in the hypertrophic zone, the needles measured about 1000 A. in length, suggesting that they had increased during maturation; the needles were pointed at one end and were of considerable electron density. It was assumed that the needles represented the mineral phase; it was noted that they were randomly oriented within a cluster and appeared to arise from a central point within the cluster (Fig. 49). The intercellular material was heavily calcified two

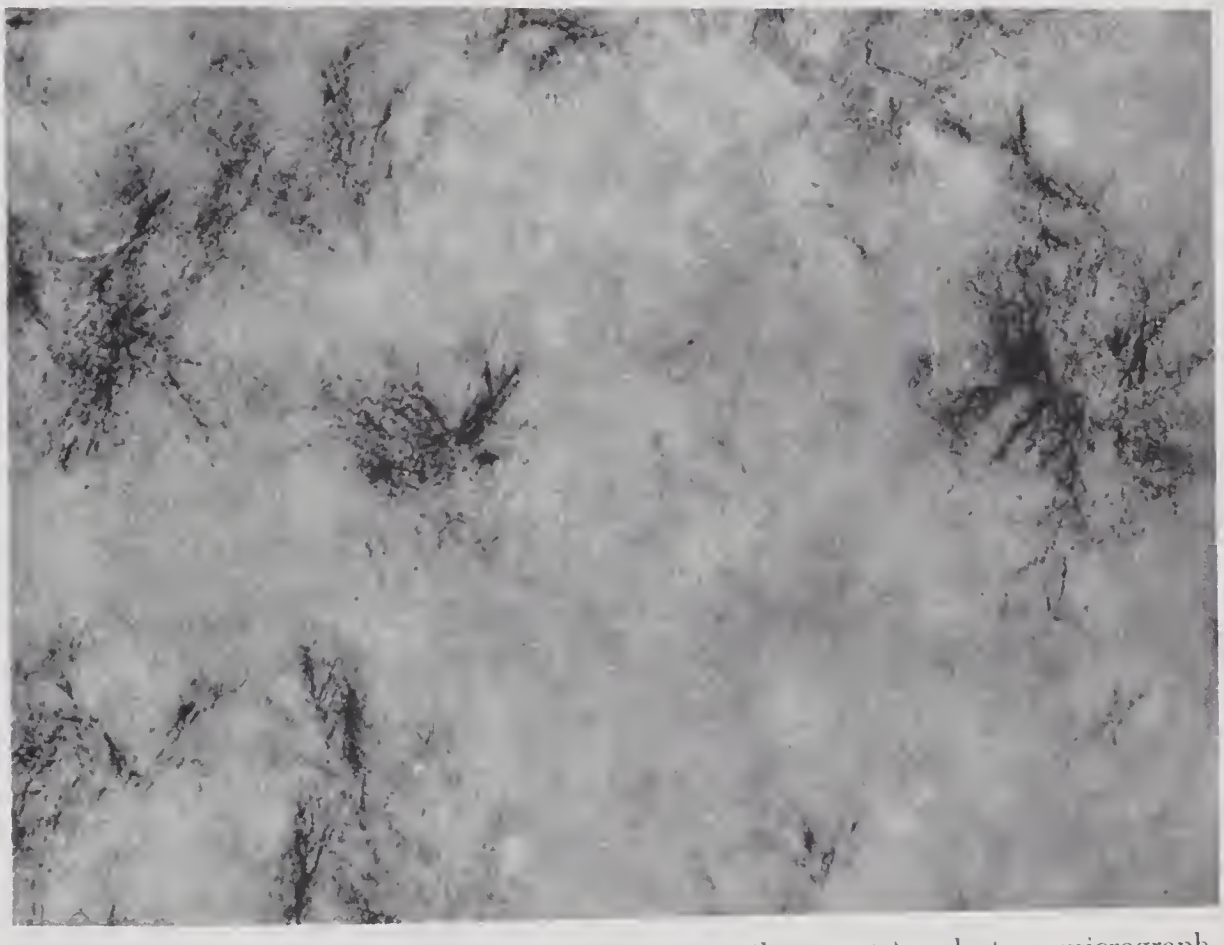


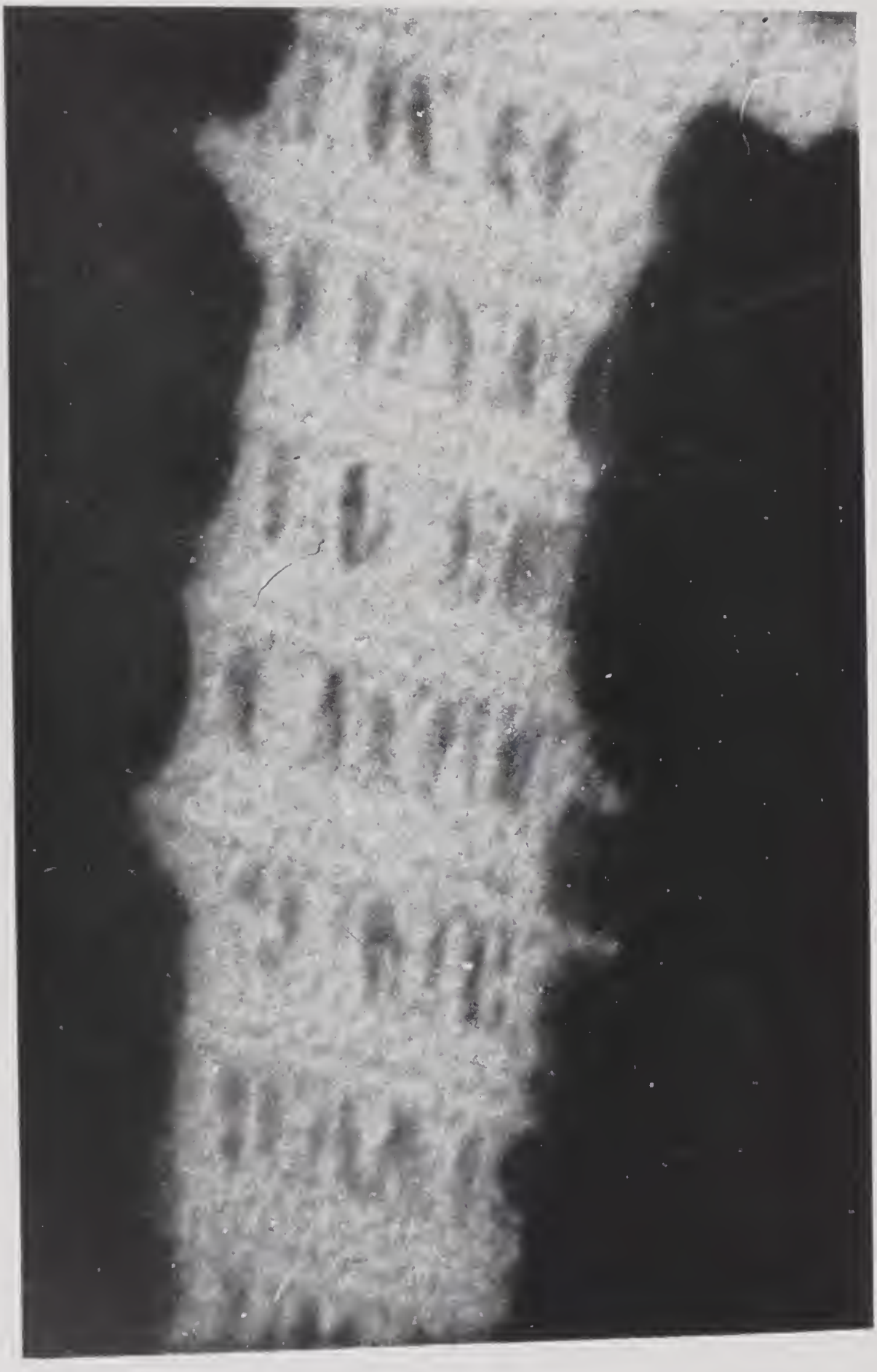
FIG. 49. The beginning of calcification in cartilage matrix; electron micrograph of a section showing the clusters of needles representing the mineral. Magnification: × 65,000.

cell layers lower, and individual clusters of the needles were contiguous one with the other. Chemical analyses of different regions of the long bone rudiments at various ages by Fitton Jackson (1960) showed that the ratio of collagen to ash in the hypertrophic zone of young chicks was about the same as that found in periosteal bone of 17-day embryos, where electron diffraction had shown that hydroxyapatite was present. The content of hexosamine, taken as a measure of the mycopolysaccharide present in the cartilage, dropped precipitously in the hypertrophic cartilage when calcification commenced, whereas the collagen content re-

mained fairly eonstant; it was noted also that the adjacent epiphyseal plate eontained the highest amount of hexosamine at a stage just prior to the onset of gross ealeifieation in the hypertrophic zone. Dziewiatkowski *et al.* (1957) reeorded a similar shift in their analyses of mammalian rudiments. At present it is open to question whether the change in the total amount of hexosamine present within the eartilage matrix may be correlated with proeesses involved in ealcifieation.

3. *The Initial Interrelationship between Collagen and Mineral*

The localization of particles in relation to the specific region of each period in the collagen fibrils in embryonie bone implies that the initial point for the formation of the apatite erystals oceurs here. The elegant experiments of Glimcher *et al.* (1957) showed that fibrils, preeipitated from solutions of extracted collagen and showing the 640 A. periodieity, ossified to the extent that small particles were seen to be associated with the fibrils but orientation was lacking. The partieles lay within the fibrils but did not appear to be so precisely located in relation to one partieular part of the collagen periodic structure as in embryonic bone. A most important observation was that collagen solutions preeipitated in fibrous array other than that of the native organization did not ossify under the experimental conditions used. These findings stressed the importance of the macromolecular organization that produeed the charaeteristic 640 A. period in the process of crystal formation of bone. The recent suggestion by Hodge (1963) on the manner of alignment of tropocollagen particles into fibrous arrays indicates that holes occur at precise points on macromolecular aggregation; analyses by Hodge showed that the position of holes coincides with the localization of crystals in embryonic bone and may be eoneerned in the induction of crystal growth. If another substanee is present in this region of the eollagen fibril it may well be eoneerned in the initial reaction. It is interesting that Glimeher (1959) found that fibrils from native collagen-rieh tissues failed to mineralize under physieoehemical eonditions identical to those used in testing the activity of the native-type fibrils reconstituted from the same tissues; if the tissues were treated first by either salt extraction or enzymatic digestion, the same tissues readily mineralized. These findings supported the idea that a constituent or eonstituents of the ground substance may be eoneerned in the inhibition of mineralization. Further support for this hypothesis is provided by the observation that appropriate solutions of eollagen mineralize quite readily in the presence of various amounts of mucopolysaccharides, but fail to react when a small proportion of proteinpolysaccharide is present (Hamerman and Schubert,



1962). If proteinpolysaccharides are associated with the regions of holes in the collagen fibrils (Fig. 50), it is possible that their presence and subsequent removal may act as a controlling factor of initial seeding points for the hydroxyapatite. Glimcher (1961) has provided evidence also that ε-amino groups may be concerned in this process.

Once the initial inductive phenomenon has been accomplished, spontaneous crystal growth must occur by the stepwise addition of calcium and phosphate ions; it is possible that the phosphate may be supplied via the cell whereas the calcium is present in the extracellular phase; certainly major crystal growth seems to occur at some distance from the cell both in cartilage and bone. Although a precise arrangement of initial seeding sites in relation to collagen has not been observed in cartilage, it is possible that the mechanism is identical to that which occurs in bone; Fitton Jackson (1960) has pointed out that the further development of crystal growth and alignment may reflect the original availability and arrangement of the initiation sites within the collagen fibrils and the orientation of the fibrils with respect to each other. Evidence has accrued recently that pyrophosphate may be concerned in the control of the mineralization process, since it appears that pyrophosphate may have to be destroyed *in loco*, possibly by a pyrophosphatase before hydroxyapatite precipitation can occur, it being assumed that the correct physiological concentration of calcium and phosphate is available (Fleisch and Neuman, 1960; Fleisch and Bisaz, 1962).

V. Some Catabolic Processes of Connective Tissue

The important physiological process of tissue breakdown occurs in many if not all connective tissues throughout life, as is evidenced by the metabolism of various constituents; for example, the mucopolysaccharides of the ground substance demonstrate a turnover rate of about 7–10 days, but the collagen moiety in many tissues is relatively inert. There are several instances, however, in which collagen is normally degraded very rapidly—for example, during bone resorption, metamorphosis, and the involution of the uterus after parturition (e.g., Harkness *et al.*, 1956), when about 90% of the original collagen is broken down, 50% being lost during the first day. No relationship between proteinase activity and collagen release, however, has been found in appropriate homogenates (Woessner, 1959). Until recently little was known about the mechanisms

Fig. 50. Collagen fibril teased from the articular cartilage of a 5-day chick; negative-stained preparation. Note the arrangement of the "holes" in the main interband zone of each period. Magnification: × 440,000.

involved in resorption, but the elegant demonstration by de Duve and associates (e.g., de Duve *et al.*, 1955) of lysosomes containing acid hydrolases in many cell types has provided an insight into physiological processes of breakdown. These studies have aided greatly various investigations of enzymatic systems possibly involved in the degradation of connective tissue.

A. Embryogenesis, Regeneration, and Metamorphosis

Many studies of rapidly growing systems indicate that a delicate balance must be achieved between the synthesis and the breakdown of various cellular products. Earlier work on developmental systems implicated proteolytic enzymes, but whether breakdown resulted from the activation of circulating enzymes normally inactive, or by the local production of activated enzymes by the cells, was open to question. Methods devised to demonstrate the intracellular localization of acid phosphatase, however, indicated that some cells were concerned in either the production or storage of a hydrolytic enzyme. Although the occurrence of digestive vacuoles within, e.g., ameba (Horning, 1926) had long been known, it was not clear how intracellular protection against enzymatic degradation was achieved.

Glucksmann (1951) made a detailed study of cell death, particularly in relation to embryogenesis, and stressed its occurrence throughout life; he discussed whether cell death might be due to excessive enzymatic activity leading to a release of degradative enzymes into the local environment. Saunders (1960) has demonstrated that there is a direct relationship between regions of cellular necrosis and the development of the limb bud.

Since the observation of Oreckwitsch *et al.* (1935) that catheptic activity changed during the normal regeneration of the tails of amphibian larvae, the query has been raised whether the enzyme was concerned in synthesis or in breakdown. In a detailed study of tail regenerates of *Xenopus* larvae 25–30 mm in length, Deuchar *et al.* (1957) showed that the catheptic specific activity was much higher in the regenerating tissue than in either the stump or the normally growing tails between 5 and 15 days and that it coincided with the greatest increase in total nitrogen and dry weight. Furthermore, study of the homogenates from 8-day specimens showed that the high specific activity of cathepsin was inherent in the regenerating tissue. Since Webber (1957) had shown that the catheptic activity was 22 times higher in the tail tissue of *Xenopus* larvae undergoing metamorphosis than during normal growth, Deuchar *et al.* suggested that the marked increase in catheptic activity during

regeneration of the *Xenopus* tails might be interpreted as an indication of protein breakdown. Such a dissolution of protein during rapid development may aid not only in spatial reorganization of the tissue, but may provide also necessary additional supplies of amino acids and peptides for use in subsequent formative processes.

The considerable catheptic activity present in the tails of amphibian larvae, the observation by Novikoff (1960) that lysosomes, as evidenced by staining for acid phosphatase, were abundant in the relevant cells during resorption of the tails, and the work on the degradation of cartilage (see Section V, C) led Weissmann (1961) to postulate that if cathepsin or other acid proteases were localized within lysosomes, then treatment of the amphibia with excess vitamin A alcohol in earlier stages of development should lead to the release of hydrolytic enzymes and hence early resorption of tail tissue. Weissmann's experiments showed that, after the administration of excess vitamin A alcohol to young larvae of *Xenopus laevis*, marked alterations occurred in the connective tissue, including loss of metachromasia in the ground substance followed by rapid resorption of the tails prior to metamorphosis.

An elegant approach to the study of mechanisms involved in connective tissue breakdown has been made by Gross and Lapiere (1962). Various tissue fragments from anuran tadpole were grown on fibrous gels formed by thermal reconstitution of purified C^{14}-labeled collagen extracted by neutral salt from the skin of young guinea pigs. The collagenolytic activity of the various explants was detected by measuring the increased area of lysis that formed around the explants during cultivation and by estimating the release of C^{14}-labeled hydroxyproline from the substrate collagen gels. Gross and Lapiere found that the skin, gut, and gills of tadpole had relatively strong collagenolytic potency that was capable of degrading the collagen gel to dialyzable peptide fragments at neutral pH and at 27°C. Control experiments demonstrated that the collagen gels were not lysed by cathepsin and other common proteolytic enzymes. The authors noted that the active tissues were those that undergo severe remodeling during metamorphosis. Nagai *et al.* (1963) recently reported that they have isolated an enzyme system which degrades collagen to peptides at physiological pH and temperature from the liquefied medium after cultivation of the amphibian tissue.

Woessner and Boucek (1961) have studied the activity of various enzymes during the development of connective tissue in subcutaneous implants of polyvinyl sponge in female rats. They found that the activity of acid phosphatase, β-glucuronidase, and a proteinase (pH 3.2) continued to increase when the DNA and collagen content had reached their

maxima, the rates being essentially parallel for the first 16 days and then showing a divergence 28 days after implantation. These results indicated that there was an increase in the intracellular concentration of the enzymes; Woessner and Boucek suggested that the three enzymes were related to the resorption of connective tissue and that, while the rate of synthesis of collagen was constant, the rate of degradation was varied. Since de Duve *et al.* (1955) had shown that these enzymes were lysosomal in origin, Woessner and Boucek suggested that lysosomes might be involved in resorption and degradative processes in connective tissue.

In view of the findings of Gross and his collaborators, it is interesting that there have been reports of collagenolytic agents identified in two mammalian tissues. Frankland and Wynn (1962) found that fractions from rat liver contained an enzyme that hydrolyzed collagen at pH 4. The highest relative specific activity was observed in a "light" mitochondrial fraction (lysosomal); the supernatant also showed considerable activity, but that in the microsomal fraction was small. Frankland and Wynn noted that after treatment of acid-soluble collagen with the lysosomal fraction, the collagen solution was no longer precipitable, 35% of the hydroxyproline was released into the supernatant, and there was a marked drop in specific viscosity with time. The activity of the preparation was lost on heating, a finding which suggested that the collagenolytic agent was probably not cathepsin C. O'Dell (1962) has identified a collagenolytic substance in a lysosomal preparation from the uterus of a near-term pig. Activity was detected at pH 3.6 at 20°C., but no activity was present in several neutral or alkaline buffers, nor at pH 3.6 by a boiled lysosomal preparation. At present there is no direct evidence that either of these preparations play a degradative role in connective tissues; nor is it known whether the lysosomal fractions are specific for collagen or whether, under certain conditions, they may attack other constituents of the intercellular material. The important fact has been established, however, that collagenolytic agents are present in both amphibian and mammalian tissues.

B. The Resorption of Bone

The resorption of bone occurs under both normal and abnormal physiological conditions; areas of erosion are frequently adjacent to regions of new deposition of bone. Many studies, referred to in the earlier section on osteogenesis, have shown that collagen fibrils are encased by hydroxyapatite crystals of unknown dimensions, but oriented in relation to the fibrils; the localization of the various constituents of the ground substance, however, is not known. By what sequence of events

does resorption occur? Von Kölliker (1873) implicated the multinucleated osteoclasts, describing a characteristic brush border of the region of the cell adjacent to the areas of erosion. He suggested that the osteoclasts secreted an acid medium capable of dissolving the bone, and pointed out that these cells must possess a specialized protective mechanism so as to withstand such an acid environment. Since the time of von Kölliker, many other studies have established that the osteoclasts are actively concerned in bone resorption.

The morphological and enzymatic properties of the osteoclast differ markedly from those of other bone cells; it is interesting that Burstone (1960) has identified acid phosphatase-containing granules in these cells; and recently a lysosomal fraction susceptible to vitamin A *in vitro* has been isolated from rat skull bones by Dingle and Fell (1963), but, since the proportion of osteoclasts is not great in such tissue, the fraction may have been derived from other bone cells.

Four major studies have been made, by electron microscopy, of bone resorption; the investigations were made of kitten material by Scott and Pease (1956), of rat bone by Gonzales and Karnovsky (1961), of human woven and chick lamellar bone by Dudley and Spiro (1961), and of embryonic chick frontal bone by Hancox and Boothroyd (1961). In zones of active bone resorption the brush border of von Kölliker, renamed ruffled border by Scott and Pease (1956), is composed of a complex of cytoplasmic processes forming deep invaginations into the cell. There was no evidence of a mechanical disruption of the bone matrix by cellular processes, but various morphological alterations were apparent in the extracellular regions. Dudley and Spiro (1961) identified also regions of the osteoclasts where the cellular outline was essentially smooth and no resorption was apparent in the adjacent matrix, and areas of osteoclastic cytoplasm containing swollen mitochondria but minimal evidence of bone resorption; this observation suggests that various zones of one osteoclast may be carrying out different metabolic activities or are in a state of comparative metabolic inactivity.

Mineralized collagen fibrils were observed in only three of these studies, but Hancox and Boothroyd reported the appearance of "naked" collagen fibrils located well within the zone of resorption; it would appear therefore that either collagen is removed before or at the time of mineral dissolution or loss of crystals occurs before the collagen fibrils dissolve. All studies, however, have observed free apatite crystals; what happens when the crystals are released from association with collagen fibrils? Scott and Pease have described crystals lying well within the cytoplasmic invaginations, and hence "giving access to the cytoplasm"

of the osteoclasts; Hancox and Boothroyd have suggested that crystals are gathered up by folds in the ruffled border of the osteoclast and thus conveyed into the interior of the cell in pinocytotic vacuoles, a mechanism that would be in agreement with their observations of the formation of such vacuoles in cultured osteoclasts. Furthermore, the folds of the ruffled border enclosed crystals and collagen fibrils. Gonzales and Karnovsky held that free crystals were phagocytosed by the osteoclasts, but added the proviso that the resultant vacuoles might in reality be in continuation with the extracellular regions. Dudley and Spiro, however, state quite clearly that in no instance were crystals observed within the osteoclasts; apparent membrane-limited vacuoles containing crystals were shown, by means of serial sections, to be but extensions of the invaginations of the cell surface. If dissolution of apatite crystals occurs outside the cell, possibly by acidic erosion, the rate of dissolution of any one crystal will depend on the rate of diffusion away from the resorption site of the ions released from neighboring crystals; such diffusion may be enhanced by the pinocytotic action of the osteoclasts, visualized in cine-films by Rose (1961) and Hancox and Boothroyd (1961). In this way the dissolved ions would be rapidly imbibed, passed through the cell, and thence into the circulatory system.

The studies of the resorption of bone have led, on the one hand, to the suggestion of the solution of collagen fibrils and interfibril material either as a primary step, or simultaneously with crystal solution; on the other hand, loss of crystals may occur first and be followed by separation of collagen fibrils, unmasking of the striated structure, and finally their dissolution. The breakdown of apatite crystals, collagen fibrils, and components of the ground substance are three distinct degradative phenomena, and each may consist of a number of separate steps; furthermore the rate of reaction of each phenomenon may differ from that of the others. A prerequisite for a logical sequence of events would be the removal, but not necessarily the dissolution, of the apatite from association with the organic matrix and the subsequent enzymatic degradation of this material, possibly aided by partial disruption of collagen fibrils in the acid environment produced by osteoclastic activity.

C. The Degradation of Cartilage Matrix

When cartilaginous limb-bone rudiments from 6–7 day chick embryos are grown in organ culture, differentiation follows the normal pattern; after cultivation in the presence of excess vitamin A, however, Fell and Mellanby (1952) demonstrated that the treated rudiments ceased to grow and became soft and gelatinous, there was a marked reduction

in metachromasia of the cartilage matrix, and cellular alterations were evident. Following the injection into young rabbits of an exogenous protease, papain, Thomas (1956) noted a rapid loss of chondroitin sulfate from all the cartilage of the body, and the collapse of the ears, 18 hours after injection. Since the effects of treatment with vitamin A and papain in these two systems appeared to be similar in many respects, comparative studies of the effects of these agents were made; Thomas *et al.* (1960) showed that hypervitaminosis A in rabbits produced changes in cartilage resembling those produced by papain, while Fell and Thomas (1960) found that papain added to limb-bone rudiments *in vitro* caused a depletion of cartilage matrix very similar to that which occurred on treatment with excess vitamin A. Fell and Thomas (1960) proposed therefore "that vitamin A may enhance the activity of a number of cellular enzymes, one of which resembles papain in its effect; such an enhanced hydrolytic activity might be due to the activation of enzymes, to their greater production or to their increased liberation through an increased permeability of the cells or their organelles."

As a result of these findings, Dingle *et al.* (1961) investigated the biochemical changes that were associated with the morphological alterations due to excess vitamin A on limb-bone rudiments in organ culture; they found that the action of the vitamin led to a loss of hexosamine, changes in RNA:DNA ratio, diminution of respiration, and increased glycolysis. These results supported the hypothesis that excess vitamin A enhanced the activity of proteolytic enzymes in the cartilaginous tissue.

Treatment of long-bone rudiments from embryonic chicks with distilled water followed by incubation at 37°C. in buffer systems produced rapid changes between pH 3 and 5 in both the histological appearance and the chemical composition of the rudiments (Lucy *et al.*, 1961). The metachromatic properties of the cartilage matrix were greatly reduced; half the hexosamine content was removed and was found to represent polysaccharide of high molecular weight. There was a concomitant loss of acid-soluble protein constituents, the amino acid composition of which indicated that it was associated with the breakdown of the noncollagenous protein moiety of the cartilage. Control specimens incubated above and below pH 3–5 failed to demonstrate similar alterations; the narrow pH optimum indicated enzymatic degradation of the tissue. Fractionation of sucrose homogenates of the embryonic long-bone rudiments, followed by homogenization of three fractions in water, demonstrated that 51% of the enzymatic activity at pH 4 was present in the "mitochondrial" fraction, 16% in the supernatant fraction, and 33% in the nuclear fraction. These results showed that hypoosmotic treatment of

limb-bone rudiments led to the release from the cells of an enzyme or enzymes which degraded the protein moiety of the proteinpolysaccharide of cartilage.

Tourtellotte *et al.* (1963) have reported briefly that an enzymatic preparation can be extracted from various cartilages with water; they showed that the pH optimum was about 4 and that the preparation split out from a proteinpolysaccharide sample obtained from cartilages a product which demonstrated electrophoretic mobility similar to that of chondroitin sulfate.

Since the results of hypoosmotic treatment resembled those produced by excess vitamin A, Fell and Dingle (1963) made a further study of the effects of the vitamin on cartilage and found that an acid protease was in fact released by excess vitamin A from cultures of long-bone rudiments. After 6 days in culture the treated explants showed advanced resorption, and in one experiment, following a 29-hour period in synthetic medium, released 275% more protease than the controls. The release of the enzyme varied in different experiments and appeared to be correlated with the severity of the morphological changes produced. It is of interest, however, that the control explants also liberated protease into the medium, and in addition the protease activity in these rudiments was considerable. There was also evidence that there was an increased synthesis of protease in the treated rudiments.

Studies by Fell and Dingle (1963) of a particulate fraction obtained from embryonic bovine and chick cartilage showed that vitamin A facilitated the liberation of an acid protease, the activity of the bovine preparation (61%) being considerably greater than that obtained from the chick cartilage (40%); some protease activity was found also in the supernatant fraction.

The work of Fell and her collaborators has led to the proposal that vitamin A when added in excess of normal requirements causes an instability in the lysosomal particles and under certain conditions results in the release of bound hydrolytic enzymes. It would appear that the mode of action of vitamin A is initially to penetrate the membrane and thus cause some instability, but it is not known yet whether the particles swell, burst, or release the enzymes through changes in the structure of the membranes.

Fell and Dingle also investigated the action of a crude protease preparation derived from rat liver lysosomes on limb-bone rudiments from 9– to 10–day–old chick embryos. The results showed that the lysosomal preparation induced a decrease of metachromasia at pH 7, whereas at pH 6 it was entirely absent and the rudiments showed a considerable

reduction in size as compared with the controls. Polysaccharide was liberated from the treated rudiments during a 12-hour period of incubation at 37°. These results showed that a lysosomal protease with an acid pH optimum degraded one component of the cartilage matrix at a neutral pH, the reaction occurring more slowly than at the acid optimum pH.

D. The Inhibition of Cartilage Degradation by Acid Protease

Fell and Thomas (1961) have investigated the influence of hydrocortisone on the degradation of limb-bone rudiments of embryonic chick and mouse grown in organ culture simultaneously with excess vitamin A. The results of the various experiments showed quite clearly that the dissolution of extracellular material normally apparent on treatment with excess vitamin A was retarded by the presence of hydrocortisone. Since recent work (Fell and Thomas, 1960; Dingle *et al.*, 1961; Lucy *et al.*, 1961) indicated that excess vitamin A increased the proteolytic activity of chondrocytes and released a protease from lysosomes particles that was capable of degrading the protein moiety of the proteinpolysaccharide, Fell and Thomas suggested that hydrocortisone might partially inhibit the synthesis, activity, or the release of the protease. An investigation by Weissmann and Dingle (1961) led to the suggestion that one action of the steroid may well be the stabilization of intracellular lysosomes against a variety of stimuli; their experiments showed that a lysosome-rich fraction from rat liver pretreated with hydrocortisone retarded the release of bound catheptic activity by ultraviolet irradiation, rather than leading to a change in proteolytic activity.

Studies of the inhibition of cartilage degradation *in vivo* have shown that the simultaneous administration of hydrocortisone and excess vitamin A acid to *Xenopus laevis* largely prevented the loss of metachromasia in cartilaginous tissue (Weissmann *et al.*, 1963). Thomas *et al.* (1963) found that under appropriate conditions cortisone also largely prevented in rabbit cartilage degradative change induced by excess vitamin A. The earlier work by Thomas (1956), however, showed that following depletion by papain, cortisone and hydrocortisone prevented the reconstitution of cartilage matrix in rabbits, and Thomas suggested that the steroid had a direct inhibitory effect on the synthesis of extracellular material. In view of the recent studies showing that cortisone has the capacity of retarding the effects of excess vitamin A *in vivo*, Thomas *et al.* (1963) have suggested that if "the lytic effect of vitamin A is not completely prevented by cortisone, and if the cortisone and vitamin A are administered over a relatively long period, there might occur both depletion and suppression of reconstitution of cartilage matrix."

It would appear, therefore, that under appropriate conditions both cortisone and hydrocortisone are capable of acting as inhibitors to cartilage degradation by protease activity, possibly by the stabilization of lysosomes and hence a reduction in the release of proteolytic enzymes.

VI. Conclusion

The foregoing discussions on various aspects of connective and skeletal tissues have perhaps raised more questions than they have answered. It is true to say, however, that a better understanding has now been reached of the nature and structure of various extracellular constituents. On the one hand, the elucidation of the configuration of the polypeptide chains of the collagen macromolecule as determined by X-ray diffraction, the demonstration that one collagen polypeptide chain has a different amino acid composition than the other two, studies of the mode of denaturation and renaturation, and analyses of the aggregation phenomenum involved in fibril formation have provided information of great value to an understanding of the mechanisms involved in fibrogenetic processes. On the other hand, studies of the various constituents of the ground substance have stressed its heterogeneity, and the structural importance of the complex of proteinpolysaccharides in some tissues. Evidence indicates a precise order in connective tissues and places emphasis on the interdependence of collagen and constituents of the ground substance at both the intra- and extracellular levels.

Knowledge of the various parts played by the cell is less clear, but certain steps concerned in the production of intercellular products have been elucidated, notably those involved in polysaccharide synthesis. In collagen synthesis, the stage at which hydroxylation occurs is still controversial, but this is only one step in the sequence of synthetic events. Of fundamental importance is a knowledge of the size of the collagen particle produced by the cell. Recent reports show that polyribosomes, structures containing several ribosomes, probably held together by messenger RNA, are the major site of peptide chain formation (e.g., Warner *et al.*, 1963). Each polypeptide chain in a tropocollagen unit contains about 1000 amino acid residues and has a chain length of about 3400 A.; if such chains are synthesized as one continuous length, the number of ribosomes required would be twelve or more. It is interesting that studies of cell fractions have shown that most of the bound-hydroxyproline of the cell was found in a large granule fraction; if all three chains of the collagen macromolecule were synthesized together, the size of the polysome would be such that it would certainly be localized within this fraction. Future work may demonstrate that such polysomes exist within

connective tissue cells and that such cell fractions can be induced to form characteristic collagen fibrils.

A major problem is the maintenance of the synthetic balance in the cell, especially during the formation of the tissues. Do the synthetic mechanisms respond directly to genetic information, brought into operation when required, or also to environmental conditions, so that the correct proportion of the different extracellular constituents are maintained? There is much evidence to support that the functional state of the cell is, at least, in part, dependent on environmental requirements. Moreover, do the various synthetic activities occur in waves, first one substance being produced and then another, or do the cells extrude the different products simultaneously in the correct amounts? The evidence indicates that cells store material destined for extracellular regions and thus suggests that even if synthesis occurs in waves, while the necessary raw products are available, the release of material from the cell depends on other factors. It is interesting that during some forms of tissue resorption, depletion of one component appears to affect the rate of synthesis of this material. Does it also affect the production of other constituents by increasing their output, for example? The mode of collagen fibril enlargement emphasizes that strictly controlled mechanisms are involved and suggests that certain constituents of the ground substance are of fundamental importance in the fibrogenetic process. That the respective cell plays many vital roles in the formation, maintenance, and resorption of connective tissues is indisputable; although collagen is the toughest fibrous protein, the elucidation of the functional mechanisms of the connective tissue cell is still tougher.

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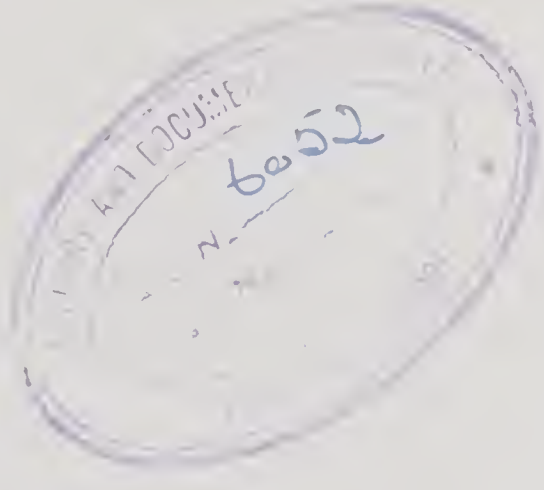

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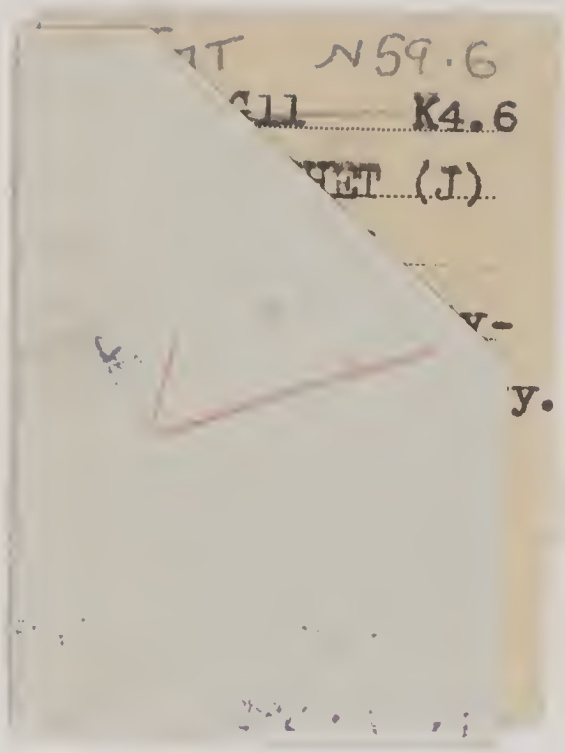
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